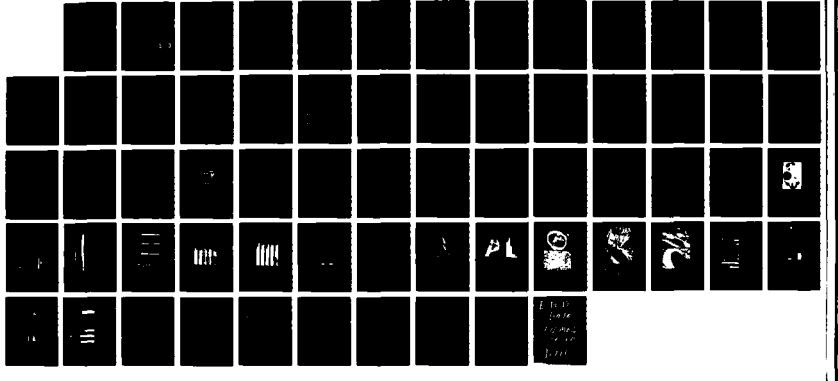
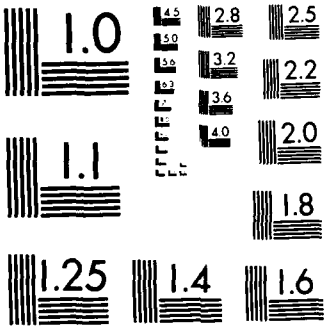
AD-A195 940 INTRINSIC CHOLINERGIC MECHANISMS REGULATING CEREBRAL 1/1
BLOOD FLOW AS A TARG..(U) CORNELL UNIV MEDICAL COLL NEW
YORK D J REIS ET AL. OCT 86 DAMD17-84-C-4105

UNCLASSIFIED F/G 6/1 NL





(2)

AD _____

INTRINSIC CHOLINERGIC MECHANISMS REGULATING CEREBRAL BLOOD FLOW AS A TARGET FOR ORGANO PHOSPHATE ACTION

ANNUAL REPORT

Donald J. Reis, Stephen P. Arneric, Costantino Iadecola

October 1986

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-84-C-4185

Cornell University Medical College
New York, New York 10021

Approved for public release; distribution unlimited

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER	2. GOVT ACCESSION NO.
3. RECIPIENT'S CATALOG NUMBER	
4. TITLE (and Subtitle) Intrinsic Cholinergic Mechanisms Regulating Cerebral Blood Flow as a Target for Organophosphate Action	5. TYPE OF REPORT & PERIOD COVERED Annual Report 9/30/85 - 9/29/86
	6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Donald J. Reis, M.D. Stephen Arneric, Ph.D. Costantino Iadecola, M.D.	8. CONTRACT OR GRANT NUMBER(s) DAMD17-84-C-4185
9. PERFORMING ORGANIZATION NAME AND ADDRESS Cornell University Medical College New York, NY 10021	10. PROGRAM ELEMENT PROJECT, TASK AREA & WORK UNIT NUMBERS
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research & Development Comm. Fort Detrick Frederick, MD 21701-5012	12. REPORT DATE October 1986
	13. NUMBER OF PAGES 61
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)	15. SECURITY CLASS. (of this report) Unclassified
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

acetylcholine, atropine sulfate, cerebral cortex, cerebral blood flow, choline acetyltransferase, cortical microvessels, electron microscopy, fastigial nucleus, GABA, HPLC, immunocytochemistry, neurotransmitter release, vasodilation, Rats, Cholinergic [illegible]

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

In year 02 we have finished one objective of demonstrating that the release of ACh is within the target area, the cerebral cortex, by establishing that the effect is blocked by local administration of atropine, and that the quantity of ACh released is small. Moreover, we have, by chemical and immunocytochemical methods, established that the source of ACh is likely to be small cholinergic nerves adjacent to cerebral vessels and/or ACh synthesized in capillary endothelium. Keywords:

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

FIGURE 2

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

SUMMARY

We sought to determine: a) whether the increase in regional cerebral blood flow (rCBF) elicited within the rat cerebral cortex (CX) by electrical stimulation of the fastigial nucleus (FN) of the cerebellum is associated with the local release of acetylcholine (ACh) and b) to establish the possible cellular source of ACh in the CX.

In the first study rats were anesthetized, paralyzed, ventilated, with arterial blood gases controlled and arterial pressure maintained within the autoregulated range. Bilateral craniotomies were performed over a standardized region of the sensory motor CX and superfusion devices stereotaxically positioned on the cortical surface. Cortical surface temperature, as well as pH, pCO_2 and pO_2 of the solutions applied to the cortex were also carefully controlled. rCBF was measured in dissected regions of frontal (FCX), parietal (PCX), and occipital cortices (OCX), caudate nucleus (CN), and hippocampus (HIPP) by the Kety principle using ^{14}C-iodoantipyrine as indicator. Resting rCBF (ml/100g/min) in unoperated control animals ranged from 70 $\pm$ 5 in HIPP to 95 $\pm$ 7 in PCX and was unaffected by bilateral craniotomies and placement of superfusion devices containing Kreb's bicarbonate buffer (vehicle) on the cortical surface. Local application of atropine (ATR, 100uM) to the right PCX via the superfusion device did not affect resting rCBF. With FN-stimulation rCBF increased bilaterally and symmetrically in all areas up to 227% in PCX. ATR application attenuated by 59% the FN-elicited increase in rCBF on the ipsilateral fronto-parietal CX, without affecting blood flow in adjacent structures. ATR did not affect cortical cerebrovasodilation produced by hypercarbia(arterial pCO_2 = 59.0 $\pm$ 1.4 mmHg). FN-stimulation resulted in a small (22%) but significant ($p < 0.05$, $N = 9$) reduction in the release of ^{3}H-ACh from the cortical surface, while supramaximal depolarization with 55 mM K^+ increased ^{3}H-ACh release by 251%.

In the second study, using immunocytochemical and neurochemical techniques, we sought to establish what proportion of the cholinergic innervation of the cerebral cortex (CX) is associated with intraparenchymal blood vessels and whether ^{3}H-acetylcholine (^{3}H-ACh) is synthesized and released by elements associated with cortical microvessels (MV). MVs and, for comparison, tissue homogenates were prepared using sucrose gradient/differential ultracentrifugation methods. Efficacy of the separation technique was indicated by the activity of gamma-glutamyltranspeptidase in the MV fraction (up to 29.2-fold enrichment) and light microscopic examination. The size of isolated microvessels ranged from 5-40 um (o.d.) with 67.7% of the vessels less than 10 um and 32.3% between 11-40 um (690 vessels measured from 4 animals). At the electron microscopic level, ChAT was found to be localized to capillary endothelial cells and nerve terminals closely apposed to the basal lamina of the microvasculature. The absolute amount of ACh synthesized (pmol ACh/100 mg wet wt.) by elements associated with cortical MVs was relatively small (2.3% total cortical homogenate activity). Other tissues similarly synthesized small amounts of ACh relative to the CX, caudate nucleus (CN, 2.4%), cerebellum (CRB, 1.4%) and liver (LIV, 3.9%). Consistent with the known extent of the cholinergic innervation of the tissues examined, the rank order of ChAT assocated for both MVs and homogenate were: CN > CX > CRB > LV. However, based on the specific activities of ChAT, cortical MVs have the remarkable capacity to synthesize ACh at rates 95% greater than cortical homogenate (59.0 $\pm$ 3.5 nmol/mg protein/40 min; n = 7), which is enriched in nerve terminals. Except for LV (+ 11%), other tissues also had remarkably high ChAT activity in MV (% above corresponding homogenate; $p < 0.05$, n = 5): CN (+269) and

CRB (+313). Inhibition of MV ChAT activity to 5% of control by the specific ChAT inhibitor, 4-naphthylvinylpyridine, and HPLC analysis of the product, indicated that authentic ACh was measured. Release of ^{3}H-ACh from MVs and, for comparison, synaptosomes was graded to K^{+}-depolarization stimulus (5-55 mM), maximal with 55 mM K^{+} and Ca^{2+}-dependent. The K^{+}-evoked release of neurotransmitter amino acids aspartate and GABA, unlike ^{3}H-ACh, was only observed in synaptosomes. This differential pattern of neurotransmitter release suggests a selective innervation of cholinergic neurons with the cortical microvasculature and that contamination of the MV fraction by non-vascularly related neurons is unlikely.

These studies indicate that: (a) increases in cortical rCBF elicited by FN-stimulation, but not hypercarbia, are in large part mediated by local muscarinic cholinergic receptors; (b) resting rCBF is not tonically affected by muscarinic receptor activation; and (c) the release of ACh from the cortical surface is, in general, reduced during FN-stimulation. These experiments, taken together with evidence of a cholinergic innervation of the cortical microvasculature, suggest that a subpopulation of cholinergic neurons innervating cortical blood vessels, or less likely, ACh released from endothelium releases a small amount of ACh during stimulation of FN to mediate, at least in part, the increase in cortical blood flow. We conclude that the synthesis and release of ACh, at the level of cortical precapillary arterioles, may provide a potent mechanism for the neural control of the cerebral circulation.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF FIGURES (continued)

LIST OF TABLES

INTRODUCTION

Neurons originating within or passing through the fastigial nucleus (FN) of the cerebellum will, when excited electrically, symetrically increase regional cerebral blood flow (rCBF) throughout the entire brain (Nakai et al., 1982; Nakai et al., 1983). The increases in rCBF are greatest in the cerebral cortex (up to 2.5-fold), occur without corresponding changes in glucose utilization (Nakai et al., 1983) and are associated with the abolition of cerebrovascular autoregulation (Reis et al., 1985). Since the increase in cortical rCBF is unassociated with changes in metabolism, the increase has been termed a primary cerebrovasodilation (Reis et al., 1985). The cerebrovascular vasodilation elicited from FN is mediated via pathways contained entirely within the CNS and, hence, represents an example of intrinsic, or central neurogenic, control of the cerebral circulation (Nakai et al., 1982; Reis et al., 1985). Since the FN does not project directly to the cerebral cortex (CX), the cortical vasodilation must involve a multisynaptic mechanism (Del Bo et al., 1982). The pathway mediating this response is still unknown, however, since the response is abolished by lesions of the basal forebrain (BF); a pathway originating in or passing through the BF is, therefore, likely (Iadecola et al., 1983a).

The global cerebrovasodilation elicited by FN-stimulation is abolished by systemic administration of atropine sulfate (Iadecola et al., 1986b). This observation indicates that the cerebrovascular response is mediated by release of acetycholine (ACh) somewhere in the brain acting upon muscarinic cholinergic receptors. This finding, coupled with the reports that ACh dilates cerebral blood vessels in vitro (Edvinsson et al., 1972; Kuschinsky et al., 1974; Lee et al., 1978) and in vivo (Gross et al., 1981; Heistad et al., 1980) suggests the possibility that cholinergic pathways within brain participate in the FN-elicited increase in cortical rCBF.

The site(s) at which ACh could act to evoke the cortical cerebrovasodilation elicited by FN-stimulation is not known. Conceivably, a site could be along the pathway between FN and CX which necessarily involves at least one synapse (Del Bo et al., 1982; Iadecola et al., 1983a; Iadecola et al., 1986b, Reis et al., 1985). On the other hand, ACh could be released in the CX itself either from afferent cholinergic fibers (about 80%) largely arising from neurons in the BF (Johnston et al., 1979; Lehman et al., 1980, Mesulam et al., 1983), from cholinergic neurons intrinsic to CX (Eckenstein et al., 1984) or even from cerebral vessels.

It is recognized that acetylcholine may phasically regulate the tone of extracranial cerebral blood vessels and, consequently, affect cerebral blood flow (CBF) (Edvinsson et al., 1972, 1977; Kuschinsky et al., 1974; Pinard et al., 1979; Scremin et al., 1986). In vitro acetylcholine (ACh) acting on muscarinic receptors dilates cerebral arteries (Duckles, 1981; Furchgott et al., 1981). Muscarinic receptors are localized to endothelial and/or smooth muscle cells at all segments of the cerebral vasculature, including large cerebral arteries (Edvinsson et al., 1977), small pial arteries and veins (Estrada and Krause, 1982) and capillaries (Estrada et al., 1983). Similarly, biochemical evidence indicate that all segments of the vasculature contain the ACh biosynthetic enzyme, choline acetyltransferase (ChAT, EC2.3.1,6) (Duckles, 1981; Estrada et al., 1983; Florence and Bevan, 1979; Goldstein et al., 1975; Parnevalas et al., 1985), and are innervated by cholinergic nerves as indicated by acetylcholinesterase staining (Edvinsson et al., 1972, 1977; Vasquez and Purves, 1979). A ubiquitous distribution of cholinergic function is suggested by a recent immunocytochemical study showing that capillary endothelial cells may contain ChAT (Parnavales et al., 1985). However, it is not

yet known whether ACh is released at smaller segments of the cerebral vasculature to mediate vasodilation.

In this study we sought to establish if the increase in cortical rCBF elicited by electrical stimulation of FN is mediated by the local release of ACh by determining whether: (1) local application of atropine to the CX abolishes the response and (2) the FN-elicited cortical cerebrovasodilation is temporally correlated with a stimulus-locked release of ACh from the CX.

Moreover, we specifically sought to establish whether ACh is synthesized and released from neural elements associated with small (< 50 um) intraparenchymal vessels of the rat cerebral cortex. Our results demonstrate at the ultrastructural level that ChAT is immunocytochemically localized to both capillary endothelial cells and nerve terminals closely apposed to the basal lamina of the microvasculature. Moreover, isolated cortical microvessels depolarized with K^+ results in the Ca^{2+}-dependent release of ^3H-ACh.

METHODS

I. ANIMALS

Studies were conducted on male Sprague-Dawley rats (290-380g) that were maintained in a thermally controlled (26-27°C) light-cycled (0700 on - 1900 off) environment, fed standard laboratory chow and given water ad lib.

II. PHYSIOLOGICAL STUDIES

A. Preparation of Animals

Methods for surgical preparation of rats for electrical stimulation of brain and measurement of rCBF are identical to those described in previous publications from this laboratory (Nakai et al., 1982) and are summarized below.

Animals were anesthetized with alpha-chloralose (40 mg/kg, s.c.) after induction with halothane (2.5% in 100% O_2) blown over the nose. Thin wall vinyl (o.d. = 0.5mm) and polyethylene (o.d. = 1.3mm) catheters were placed in each femoral artery and vein, respectively, and the trachea was cannulated.

Animals were then placed in a stereotaxic frame with the head adjusted so that the floor of the IVth ventricle was horizontal (bite bar position: -11 mm). After connecting the tracheal cannula to a small-animal respirator (Harvard Apparatus, Model 680), the animals were paralyzed with tubocurarine (0.5 mg/kg, i.m., initially; supplemented with 0.2 mg/kg hourly), and ventilated (80 cpm) with 100% O_2. Halothane was continued at a reduced rate (1%) during surgery. Continuous monitoring of arterial pressure and heart rate was done through one of the arterial catheters connected to a Statham P23Db transducer which was coupled to a chart recorder.

The lower brainstem and caudal half of the cerebellum were exposed by an occipital craniotomy. After completion of the surgery, halothane was discontinued. A small volume (about 0.2 ml) of arterial blood was sampled after surgery for measurement of pO_2, pCO_2 and pH by a blood gas analyzer (Instrument Laboratories, Model Micro 13). In control animals arterial blood gases were maintained so that pO_2 was greater than 100 mmHg, pCO_2 = 33-38 mmHg, and pH = 7.35-7.45 (see Table 1). Adjustments were made by changing the stroke volume of the ventilator.

B. Electrical Stimulation of FN and Recording of Electrocorticogram

The FN was stimulated with cathodal current delivered through monopolar electrodes fabricated from Teflon-insulated stainless steel wire (150 um, o.d.), carried in 28-gauge stainless steel tubing and exposed at the tip for 100 um. The anode (ground) was a clip attached subcutaneously to neck muscle. Electrical pulses were generated by a square-wave stimulator (Grass, Model S88) and constant current was passed through a photoelectric stimulus-isolation unit (Grass Model PSIU6). The stimulus current was measured by continuously displaying on an oscilloscope the voltage drop across a 10 ohm resistor.

The electrode was mounted on a stereotaxic micromanipulator and lowered into the cerebellum with a posterior inclination of 10°. The area of the FN from which an increase in CBF (Nakai et al., 1982) could be elicited was identified by localization of the most active site for the fastigial pressor response (FPR; Miura

and Reis, 1969). The area of the cerebellum explored extended 4.8-5.2 mm anterior to, 0.6-1.0 mm lateral to, and 2.0-0.5 mm above the calamus scriptorius, the stereotaxic zero reference point. To localize the most active area of the FN for the FPR, the electrode was moved in steps of 0.5 mm while stimulating with 8 sec trains of 0.5 msec duration pulses, at a frequency of 50 Hz and intensity of 20 uA. When the FPR was elicited, the threshold current, defined as the stimulus current which increases arterial pressure 10mmHg, was determined. For blood flow and ACh release experiments, the stimulus current was set at five times threshold.

The electrocorticogram (ECoG) was recorded ipsilateral to the side of the stimulation. Recording was bipolar between a pair of stainless steel Teflon-coated wires fixed to the outside of the superfusion device. The electrodes contacted the dura firmly, while ground was a metal clip placed subcutaneously in the neck. The ECoG signal was fed into an AC amplifier (Grass, Model 7P511) and displayed on a channel of the polygraph.

C. Cerebral Blood Flow Measurement

As described in detail elsewhere (Nakai et al., 1982), CBF was measured using ^{14}C-iodoantipyrine (IAP) as indicator (Sakurada et al., 1978). Tissue concentrations of IAP were obtained by the tissue sampling technique (Ohno et al., 1979). The brain:blood partition coefficient used was 0.8 (Sakurada et al., 1978).

Arterial concentration-time curve of iodoantipyrine

4-(N-methyl-^{14}C) iodoantipyrine in ethanol (New England Nuclear, 40-60 mCi/mmol) was dissolved in about 1 ml of normal saline after elimination of ethanol. Animals received 2000 units of heparin i.v. approximately 10 min prior to IAP infusion. The indicator was infused (5 uCi/100g of body weight) at a constant rate over 30-35 sec through a femoral venous catheter by an infusion pump (Harvard Apparatus, Model 940). Simultaneously, about 50 ul of arterial blood was sampled every 2-5 sec through the femoral arterial catheter in order to obtain the arterial concentration-time curve of IAP. The sampling catheter was kept short (5 cm) to more accurately reflect the arterial concentration-time curve of IAP. Aliquots (40 ul) of arterial blood were transferred to scintillation vials containing 1 ml tissue solubilizer (Protosol, New England Nuclear: ethanol, 1:1 v/v). The blood mixture was incubated at 60°C for 1 hour, decolorized with 30% hydrogen peroxide, and mixed with 15 ml of Biofluor (New England Nuclear). Radioactivity was measured by a liquid scintillation spectrophotometer (Beckman, LS 5801) and corrected to disintegrations/min (d.p.m.) using an external standard.

Measurement of tissue concentration of IAP

Approximately 30 sec following the start of the infusion of IAP, the animal was killed by a bolus injection of 1 ml of saturated KCl into the femoral vein catheter. The brain was rapidly removed, placed in liquid freon (-30°C) for 10 sec, and put on an ice-cold glass plate. Right and left samples of 5 brain regions were dissected: parietal CX (beneath, medial and lateral to the cortical superfusion device); frontal CX (2-4 mm rostral to the superfusion device); occipital CX (2-4 mm caudal to the superfusion device); caudate nucleus and hippocampus. Tissue samples were transferred to tared scintillation vials and the tissue weights determined. After solubilization of the tissue in 1 ml of Protosol, 10 ml of scintillation cocktail (Econofluor, NEN) was added and the samples counted.

Calculation of CBF

CBF (ml/100g/min) was calculated by obtaining a relationship between CBF

and tissue concentration of IAP, using a computerized approximation of the equation developed by Kety (1951).

D. Experimental Protocol

Stimulation of Fastigial Nucleus for Measurement of rCBF and ACh Release

One hour after completion of surgery, a stimulating electrode was lowered through the cerebellum and positioned in the most "active" portion of the FN, as described above. Care was taken during exploration to avoid large abrupt changes in AP and to maintain AP within the autoregulated range of CBF for the rat (80-150 mmHg) (Hernandez et al., 1978). The electrode was left in place at the active site and blood gases were carefully adjusted. At this point in the protocol the animal was either prepared for cortical application of drugs and measurement of rCBF, or prepared for measuring the release of ^{3}H-ACh.

Cortical Application of Atropine for rCBF Measurement

In these experiments the cortical superfusion device was carefully placed on the pial surface 30 min prior to rCBF measurement. Either vehicle (Kreb's-bicarbonate buffer) or atropine sulfate (100 uM) was superfused for 10 min prior to electrical stimulation of FN. For rCBF measurement the FN was stimulated with an intermittent stimulus train (1 sec on/1 sec off; pulse duration, 0.5 msec; frequency, 50 Hz). During the first 2-4 min of stimulation the intensity of the stimulus was gradually increased to reach 5x the threshold current while the evoked rise in AP was concurrently reduced by slow, controlled withdrawal of blood (4-6 ml) to maintain the AP in the autoregulated range. FN stimulation continued for 7-10 min during which the AP remained stable and the blood gases were adjusted (Fig. 3). At the end of this phase ^{14}C-IAP was infused for 30 sec, the animals killed, and the amount of radioactivity in the arterial blood and brain tissue determined.

Release of ^{3}H-ACh From Cortical Surface

For these studies, a stimulating electrode was positioned in the FN and the cortical superfusion device placed shortly following the completion of the surgery. Just prior to the placement of the superfusion device, nerve terminal stores of the cholinergic innervation to the primary motor CX were prelabeled with ^{3}H-methylcholine (1.5 uCi/1 ul; specific activity 80 uCi/nmol, New England Nuclear). The ^{3}H-methylcholine was microinjected at a depth of 1.5 mm below dura through a 70 um (o.d.) glass pipette at a rate of 200 nl/min with the aid of a micromanipulator and an air-driven mechanical valve system (Amaral and Price, 1983). When the cortical superfusion device was put in place, the pial surface was superfused with modified Kreb's-bicarbonate buffer (100 uM physostigmine) at a rate of 10 ul/min. Superfusate was collected in 8 min epochs for 2 hours into microcentrifuge tubes containing 8 ul 1.0 N perchloric acid. Immediately following the experiment the samples were frozen (-20^{o}C) for later analysis of ^{3}H-ACh.

Release of ^{3}H-ACh was quantified during: (a) resting conditions (i.e., spontaneous release); (b) during electrical stimulation of FN; and (c) during depolarization of the cortical surface with 55 mM K^{+}. Increased potassium concentrations were compensated by an equiosmolar decrease in sodium concentration.

Preliminary experiments (N=5) demonstrated that one hour following microinjection of ^{3}H-methylcholine greater than 99% of the tritium was restricted to the right parietal cortex (data not presented). This suggested that the release of ^{3}H-

ACh measured during the experiments was from the target area of the CX and not from adjacent structures. Moreover, to discount the possibility that the procedure of microinjecting ^{3}H-choline into the parietal CX caused a paralysis of the vasculature, we concurrently measured the release of ^{3}H-ACh and rCBF in the targeted area. In five rats, FN stimulation elevated rCBF in the parietal CX to 179±16 ml/100g/min, which was not significantly different from operated control animals (188±21 ml/100g/min). Finally, we verified the viability of the underlying cortical area to generate a normal ECoG that was responsive to electrical stimulation of distant brain regions. As previously demonstrated (Underwood et al., 1983), electrical stimulation of the FN was characterized by a stimulus-locked, regular, slow-wave activity, without a substantial change in amplitude (Fig. 4A). Also, electrical stimulation of the pontine reticular formation (4.0 mm ventral to FN) elicited the classical stimulus-locked low amplitude fast activity (Fig. 4B) identical to the desynchronization response first described by Moruzzi and Magoun (1949). Lastly, application of K^+ did not result in spreading depression since the ECoG shifted to a lower amplitude pattern of similar frequency (Fig. 4C) and not to the flat or desynchronous pattern which is typical of spreading depression.

E. Calculations and Statistics

Data were calculated as the mean ± S.E.M. Routinely, data were expressed as a percent of the control (vehicle) response for ease of graphical presentation. However, the statistical analyses were always performed on the absolute values of the data obtained from each experimental group. Data were analyzed by analysis of variance (ANOVA) with treatment differences being detected by Duncan's new multiple-range test. The criterion of statistical significance was $p < 0.05$.

Release values for *in vivo* experiments were calculated as the amount of neurotransmitter released per cm^2. For the *in vivo* release experiments, basal release is defined as the average of the two epochs (br_1, br_2) measured immediately prior to the two epochs during the evoked release (Er_1, Er_2). An estimate of the predicted spontaneous release (Sr) occurring with the *in vivo* release experiments was determined during the period of interest as a linear interpolation between the basal release (i.e., $\frac{br_1 + br_2}{2}$) and the average of the two poststimulus release samples (i.e. $\frac{pr_1 + pr_2}{2}$). Thus spontaneous release was $Sr = \frac{br_1 + br_2 + pr_1 + pr_2}{4}$, while the evoked release was $\frac{Er_1 + Er_2}{2}$. For the given experimental groups, the mean ± S.E.M. of Sr and Er were calculated and compared by ANOVA.

III. NEUROCHEMICAL STUDIES

A. Release of Acetylcholine from Cerebral Cortex and Brain Slices

Cortical Superfusion Device

A schematic of the device used to apply atropine to the cortical surface and collect superfusate during the release experiments is shown in Fig. 1A. For the placement of this device, holes (2.5 mm, o.d.) were drilled bilaterally over the parietal CX in an area 2.0-4.5 mm lateral and +0.5 to -2.0 mm AP to bregma. Following incision of dura with a needle tip and retraction with forceps, the device was stereotaxically positioned on the underlying sensory motor CX. Placement was made using microscopic examination to avoid occlusion of pial vessels. The cortical surface and superfusate temperature was clamped at 37 ± 0.5°C with the aid of a surface thermistor and overhead heating lamp coupled through a servo-mechanism (YSI Instruments, model 73A). Solutions filling the device and

contacting the CX were bubbled with 95% O_2: 5% CO_2 immediately before each experiment to carefully control for pH (7.3-7.4), pCO_2 (30-40 mmHg) and pO_2 (300-500 mmHg) (refer to Table 2); then superfused over the pial surface at 10 ul/min with an infusion pump (Harvard Apparatus model 940). The superfusate consisted of a modified Kreb's-bicarbonate buffer containing physostigmine to inhibit ACh degradation (in mM: NaCl, 118; $CaCl_2$, 1.2; KCl, 4.8; $MgSO_4$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 25; D-glucose, 11; choline chloride, 0.001; physostigmine, 0.1). Figure 1B shows the region of the CX affected by the superfusion device. The area of the cortical surface exposed was 0.018 cm^2.

Brain Slice Preparation

Preliminary studies were done in vitro to establish: (a) whether the release of ACh evoked by K^+-depolarization could be measured from small regions of the CX, and (b) what concentration of K^+ is required to evoke the maximal release of ACh from this restricted area of cortical tissue.

Rats were killed by decapitation. The brains were rapidly removed and placed in ice-cold Kreb's bicarbonate buffer containing 100 uM physostigmine. Slices of CX were obtained from tissue dissected 1.5 mm rostral to and 0.5 mm caudal to the anterior commissure. Coronal sections (0.3-0.5 mm thick) were prepared with a McIlwain tissue chopper and bisected into right and left halves. Up to 8 hemi-sections of CX were prepared from each brain. To correct for possible regional variations in release, all of the samples of CX from one brain were pooled, and two slices were randomly assigned to each treatment. Average wet tissue weight for the slices was 19.9 $\pm$ 0.8 mg (n=12).

Release of ^{3}H-ACh was studied using modifications of the radiochemical method described by Hadhazy and Szerb (1977). Briefly, two slices were incubated for 20 min at 37°C in Kreb's bicarbonate buffer gassed with 95% O_2: 5% CO_2, which also contained 20 uCi/ml of (^{3}H)-methylcholine (New England Nuclear, 80 Ci/nmole). The slices were then transferred at 5-min intervals, through a series of 1.5 ml micro centrifuge tubes (Eppendorf) containing 1.0 ml of gassed Kreb's bicarbonate buffer. Neurotransmitter release was evoked by exposing the tissue to potassium (5-55 mM) for 5 min. Increased potassium concentrations were compensated by an equiosmolar decrease in sodium concentration. Immediately following exposure to the brain slices, the buffer was stored at -20°C for later analysis of ^{3}H-ACh.

In vitro studies showed that spontaneous efflux reached a steady-state by 10-15 min (Fig. 2A). Depolarization with 55 mM K^+ in the presence of Ca^{2+} elicited a marked stimulus-locked release of ^{3}H-ACh. Our previous experiments (Arneric and Reis, 1986a) have established that this K^+-evoked release of ^{3}H-ACh is abolished when extracellular Ca^{2+} is removed. The evoked efflux of ^{3}H-ACh was dependent upon the concentration of potassium (5-55 mM) and was supramaximal at 55 mM K^+ (Fig. 2B). These experiments established that release of ^{3}H-ACh can be accurately measured from small tissue samples of the CX and that the depolarization stimulus required to evoke the maximal release of ^{3}H-ACh is 55 mM K^+.

B. Preparation of Microvessels and Synaptosomes

Intraparenchymal vessels were separated from brain. These ranged from the largest penetrating arterioles (50 um) to the small capillaries (5 um) (Nakai et al., 1981). Others have shown preparations enriched in rat cortical capillaries (i.e.

90% of the vessels have diameters < 10 um) contain very little of the ChAT activity found in whole homogenates (2-5%, see Goldstein et al., 1975; Santos-Benito and Gonzalez, 1985). In order to include other vascular segments which may be more densely innervated by cholinergic neurons, the method described by Reinhard and co-workers (1979) was used. This procedure was shown previously to result in a pellet that contained MVs ranging from 5-50 um, o.d.

Rats were stunned and killed by decapitation. The brains and livers were rapidly removed and placed in ice-cold modified Kreb's bicarbonate buffer containing (in mM): NaCl, 118; KCl, 5; $MgSO_4$, 1.2; $NaHCO_3$, 25; D-glucose, 11; NaH_2PO_4, 1.2; $CaCl_2$, 1.2; choline chloride, 0.001; and physostigmine, 0.1. All subsequent procedures were performed at 4°C. The pia-arachnoid membranes were carefully removed from the brain. Portions of the parietal cortex (CX), caudate nucleus (CN), cerebellum (CRB) and liver (LIV) were removed and homogenized (10 strokes) in 10 vols of 0.32 M sucrose using a Potter-Elvehjem tissue grinder. The homogenate was centrifuged at 900 x g for 10 min. After decanting, the supernatant (S_1) was centrifuged at 27,000 x g for 20 min to obtain a crude synaptosomal pellet (P_2). Fractions S_1 and P_2 are enriched in resealed nerve terminals, i.e. synaptosomes (Gray and Whittaker, 1962; Whittaker, 1969). The P_1 pellet was resuspended in 0.25 M sucrose (0.75 ml) and layered over a discontinuous sucrose gradient consisting of 1.25 ml of 1.0 M sucrose (middle layer) and 3.0 ml of 1.5 M sucrose (bottom layer) and centrifuged at 65,000 x g for 45 min in a Beckman SW-55 rotor. The resultant pellet containing the microvessels (MV) or the synpatosomes (P_2) were reconstituted to their original volume using gassed (95% O_2: 5% CO_2) buffer, pH = 7.4. These fractions were either assayed the same day or frozen at -20°C for future analysis.

C. Biochemical Assays

Measurement of Radiolabeled ACh

Release of ^{3}H-ACh was measured using modification of the radiochemical method of Hadhazy and Szerb (1977).

^{3}H-choline and ^{14}C-acetylCoA were used as precursors for the synthesis of radiolabeled ACh after prelabeling of cortex in vivo, brain slices, or preparations of synaptosomes and/or microvessels. It was necessary to verify that the radioactivity released from the tissue was authentic ACh. This was established by two methods: an enzymatic liquid-cation exchange method modified from Briggs and Cooper (1981) and HPLC separation of ^{3}H- or ^{14}C-ACh by the methods of Potter et al. (1983).

Radiolabeled ACh was routinely separated after incubation using the enzymatic liquid-cation exchange method. 200 ul of the superfusate was incubated for 30 min at 37°C in a final volume of 400 ul containing (in mM): NaH_2PO_4, 100 (pH = 8.5); ATP, 0.36; $MgCl_2$, 6.0; and 50 ug/ml choline kinase. The reaction was stopped by placing the sample on ice, adding 0.5 ml of tetraphenylboron in 2-heptanone and the microcentrifuge tube shaken vigorously. The samples were centrifuged, the organic layer containing radioactive ACh removed and the extraction repeated with an additional 0.5 ml of tetraphenylboron in 2-heptanone. The radioactivity in the organic and aqueous layers was measured by liquid scintillation counting methods (LS-5801, Beckman Instruments). Preliminary studies have shown that greater than 99% of the radioactivity in the organic phase corresponds to radiolabeled ACh (Arneric and Reis, 1986).

Further confirmation of the identity of the radioactivity was accomplished by using HPLC analysis. Samples were injected with a Model U6K injector (Waters Associates) onto a 10 um ODS column (4.6 x 100 mm). The mobile phase was 96% 0.01 M sodium acetate (pH = 5.0) containing 30 mg/liter 1-octanesulfonic acid, sodium salt (SOS), and 4% acetonitrile. The flow rate was 0.8 ml/min produced by a single-stage miniPump (Laboratory Data Control) operating at pressure of 250-350 psi. The identity of unknown metabolites was determined by matching the retention times to radioactive standards. Before injection of the biological samples, each sample was evaporated and reconstituted in 30 ul of mobile phase. As for the standards, 20 ul aliquots were analyzed. Fractions were collected at 0.25 min intervals to resolve the peaks of radioactivity; retention times of 2.75 min for ^{14}C-acetylCoA, 4.5 min for ^{3}H-choline and 6.75 min for ^{14}C-ACh were observed.

Synaptosomes or microvessel fractions were incubated for 20 min at 37°C in Kreb's bicarbonate buffer gassed with 95% O_2:5% CO_2, containing 100 uCi/ml (^{3}H)methylcholine (New England Nuclear, 80 Ci/mmol). Using a vacuum manifold, aliquots of synaptosomal (200 ul) or MV (500 ul) suspensions (approximately 0.2 - 0.5 mg protein) were deposited on Metricel Membrane Filters (25 mm o.d.; 0.45 um; Gelman Sciences, Inc.). The filters were then washed three times with Kreb's bicarbonate buffer to remove the excess ^{3}H-choline. The filters containing the tissue were transferred to glass scintillation vials containing Kreb's bicarbonate buffer (1 ml) warmed to 37°C. Neurotransmitter release was measured after exposing the tissue to K^+ (5 - 55 mM) for 10 min. Increased K^+ concentrations were compensated by equimolar reductions in Na^+ concentration. To determine the Ca^{2+}-dependency of neurotransmitter release, the Kreb's bicarbonate solution was modified by replacing Ca^{2+} with Mg^{2+} (1.2 mM) during the K^+-depolarization. To stop the release process, the scintillation vials were placed on ice and the incubation media was filtered through disposable syringe filter assemblies (13 mm o.d.; 0.45 um; Gelman Sciences) into microcentrifuge tubes containing 100 ul of 1 N perchloric acid. Samples were then stored at - 20°C for later analysis of ^{3}H-ACh and AA.

Measurement of Endogenous Amino Acids

Endogenous gamma-aminobutyric acid (GABA), aspartate (Asp) and glycine (Gly) were measured by an HPLC technique based on the method of Jones et al., 1981. This technique involves precolumn derivatization of the amino acids with O-phthalaldehyde, separation by reverse-phase chromatography and fluorescence detection of the AA derivatives. The details of this technique have been reported previously (Arneric et al., 1986) demonstrating a limit of detection of 50 fmol/10 ul of injectate.

Measurement of Choline Acetyltransferase (ChAT) Activity

ChAT activity was measured using the method of Fonnum (1975). Suspensions (25 ul aliquots) of whole homogenate (HOM), MVs, or synaptosomes were sonicated for 5 sec in 5 mM KH_2PO_4 (pH = 7.0) containing 0.2% triton X-100 and 5 mM EDTA (2:1, suspension/buffer) just prior to use. The production of ^{14}C-ACh from ^{14}C-acetyl-CoA (58.6 mCi/mmol, New England Nuclear) was determined following a 40 min incubation at 37°C. This method has been shown to selectively extract ^{14}C-ACh, but not ^{14}C-acetylcarnitine (Tucek et al., 1987) which is the product of another acetyltransferase, carnitine acetyltransferase (EC. 2.3.1.7) (Bresolin et al., 1982).

In some experiments the selective inhibitor of ChAT, 4-naphthylvinylpyridine (NVP), was used to confirm that the activity measured was the result of ChAT and not some other enzyme. For these experiments, it was necessary to prepare a soluble form of the enzyme. To do so, fractions were sonicated in buffer as before, then centrifuged at 109,000 x g for 1 hr (SW-55 rotor, Beckman). An aliquot of the supernatant was incubated in the presence and absence of 100 uM NVP for the determination of ChAT activity.

Other Enzyme Activities

Alkaline phosphatase and gamma-glutamyl transpeptidase (γ-GTP) activity was measured in samples of MV and synaptosomes. Tissue samples were sonicated in 5 mM KH_2PO_4 (pH = 7.0) for 5 sec and aliquoted for each assay. Alkaline phosphatase was measured by modification of the method of Williams et al. (1980). Tissue samples (100 ul) were incubated at 37°C for 20 min with 5 mM $CaCl_2$, 100 mM KCl, 50 mM $MgCl_2$ and 100 mM Tris·HCl (pH = 9.0), in a total volume of 0.2 ml. The reaction was stopped by adding 0.4 ml of 1 N NaOH; then 0.6 ml of distilled H_2O (4°C). Samples were centrifuged for 2 min in a microcentrifuge; the absorbance of the supernatant was measured at 410 nm and compared with nitrophenol standards. γ-GTP activity was measured by the method of Orlowsky and Meister (1965) using 5 mM L-γ-glutamyl-p-nitroanilide (Sigma) as substrate.

The protein content of samples were measured by the Coomassie Blue dye-binding method (Bradford, 1976; Read and Northcote, 1981), with bovine serum albumin as standard.

IV. HISTOLOGY

A. Electrode Placement

Localization of electrode placements were analyzed in sections stained by thionin as described previously.

B. Morphological Analysis of Microvessels

Light Microscopy

The MV and synaptosomal fractions were routinely examined by light microscopy for composition and purity. Twenty-five ul aliquots of MV or synaptosomes were spread onto gelatin (0.5%) or poly-D-lysine (1%; MW = 150,000 - 300,000) coated slides and allowed to dry at room temperature. The tissue was post-fixed with 10% buffered formalin or 4% paraformaldehyde in 0.1 M phosphate buffer (pH = 7.4), dehydrated, rehydrated and stained with 0.75% methylene blue. Ten random fields were examined qualitatively for each preparation at a final magnification of 200x.

For quantitative analysis of the distribution of the diameters of the vascular elements a computerized image analysis system was used (Spatial Data System, Eyecom II; PDP 11/45). The slides were viewed with a Leitz microscope (at 40x) coupled to a Vidicon scanner. With a joystick-controlled cursor the diameters of the vessels were calculated at the branching points of the vascular tree (see Fig. 10A).

Electron Microscopy

a. In vitro. Aliquots (0.2 - 1.0 ml) of MVs or synaptosomes were post-fixed for 10 min with 4% paraformaldehyde in 0.1 M phosphate buffer (pH = 7.4)

contained in 1.5 ml microcentrifuge tubes. All steps of the tissue processing were completed in the microcentrifuge tubes by gently centrifuging the tissue (500 x g for 1 min), drawing off the supernatant, and adding the next reactant. The fixed tissue was washed with 0.1 M phosphate buffer (pH = 7.4), dehydrated with alchohols, placed in propylene oxide and treated with 2% osmium tetroxide for 2 hours. Pellets of the fixed tissue were embedded in Epon-812 and sections (500 Å) cut through selected portions of the pellet on a LKB microtome. Sections were collected on grids and counterstained with uranyl acetate (20 min) and Reynold's lead citrate (5 min) for examination with a Philips 201 electron microscope.

b. In Situ. Male Sprague-Dawley rats (200-250 gm) were deeply anesthetized with Nembutal (50 mg/kg, i.p.) and perfused through the heart with 0.2% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer (pH=7.4). At the termination of the procedure, the brains were removed from the skulls, cut into 4 mm wide blocks and placed in the above fixative for 30 min. The blocks were then placed in 0.1 M phosphate. The region of the cerebral cortex at the level of the caudate nucleus was coronally sectioned (30um) on a Vibrotome (vibrating microtome).

A monoclonal antiserum to ChAT was produced from rat-mouse hybridomas and tested for specificity by Boehringer-Mannheim Biochemicals (Eckenstein and Thoenen, 1983). The antiserum was localized in the tissue by a modification (Pickel et al., 1975; Pickel, 1981) of the peroxidase anti-peroxidase (PAP) method of Sternberger (1979). Briefly, this procedure consisted of a sequential incubation of the sections with: (1) a 1:40 diultion of the ChAT antiserum; (2) a 1:50 dilution of rabbit anti-rat immunoglobin (IgG); (3) a 1:100 dilution of a rat PAP complex. The diluent and washes were prepared with 1% rabbit serum in 0.1 M Tris-Saline (pH=7.6). Tissues were incubated with primary antiserum for 18-24 hours at 4°C and with IgG or PAP for 1 hour at room temperature. All reactions were carried out with continuous agitation. The PAP reaction product was demonstrated by incubation with 3,3'-diaminobenzidine (DAB) and hydrogen peroxide.

The labeled sections were postfixed 1 hour in 2% osmium tetroxide in 0.1M phosphate buffer, dehydrated in a graded series of ethanols and embedded between two plastic coverslips in Epon 812. Regions of the cerebral cortex containing ChAT-labeled processes were selected and embedded in Beem capsules. Ultrathin sections were collected on grids from the surface of the plastic embedded tissues, counterstained with 5% uranyl acetate and Reynolds lead citrate, and examined with a Philips 201 electron microscope.

RESULTS

I. RELATIONSHIP OF CHOLINERGIC RELEASE TO VASODILATION IN CEREBRAL CORTEX

A. Effect of Topical Application of Atropine on the Cortical Vasodilation Elicited by Stimulation of the Fastigial Nucleus

In animals in which the cranium was intact (n=6), rCBF ranged from 70 ± 5 in hippocampus to 95 ± 7 in parietal CX (Table 3). Values did not differ between right and left side ($p > 0.05$) and were similar to those obtained in the anesthetized rat from this (Nakai et al., 1982) and other (Sakurada et al., 1978) laboratories. After a bilateral craniotomy, application of vehicle or atropine (100 uM) to the cortical surface did not affect resting rCBF in the underlying parietal CX nor in other regions (Table 3).

Electrical stimulation of the FN in untreated rats increased rCBF bilaterally and symmetrically in all regions sampled (Fig. 3), with the greatest increases occurring in the parietal CX. Topical application of atropine (100 uM) to the right parietal cortex significantly reduced the elevation in rCBF by 55% in the ipsilateral parietal CX and by 62% in the ipsilateral frontal CX (Fig. 5) However, in the remainder of the brain the elevations in rCBF were not significantly reduced by atropine (Fig. 5).

B. Effect of Topical Application of Atropine on the Cerebrovasodilation Elicited by Hypercarbia

Arterial pCO_2 elevated to 59.0 ± 1.4 mmHg (N=5) with 5% CO_2, increased rCBF in all areas of brain to a level comparable to that obtained with FN-stimulation (Fig. 6). Atropine (100 uM) applied to the right parietal CX (n=5) had no effect on the magnitude of the cerebrovasodilation either locally or elsewhere in brain (Fig. 6). Thus, the reduction of the cortical vasodilation evoked from the FN by ATR is not a consequence of a non-specific action of the drug on cerebral vessels, and the cortical vasodilation elicited by hypercarbia does not involve a cholinergic link.

D. Effect of Stimulation of the Fastigial Nucleus on the Release of Acetylcholine from Cerebral Cortex

The observation that topical cortical application of atropine attenuates the cortical vasodilation elicited from the FN strongly suggest that ACh released locally is involved in the cortical vasodilation. We therefore sought to establish whether stimulation of the FN releases ACh from the CX.

Release of ^{3}H-ACh from the cortical surface was measured following microinjection of ^{3}H-choline into the parenchyma of the parietal CX, which initiates the local biosynthesis of ^{3}H-ACh. After the microinjection, the superfusion device was stereotaxically positioned on dura and superfusion was begun. The effect of electrically stimulating the FN or depolarizing the cortical surface with 55 mM K^+ is illustrated in a representative experiment (Fig. 7). Within 30 min of superfusion the rapid efflux of ^{3}H-ACh began to stabilize. When FN was stimulated for 16 min there was no apparent effect on the efflux of ^{3}H-ACh. In contrast, local depolarization with K^+ increased ^{3}H-ACh release over 2.5-fold. This K^+-evoked release of ^{3}H-ACh indicated that we could reliably measure

the release of ^{3}H-ACh with the experimental conditions used. The grouped data of 9 similar experiments demonstrated that FN-stimulation significantly reduced the release of ^{3}H-ACh (Fig. 8). The validity of this finding was supported by establishing that during these experiments cortical rCBF was elevated (N=5; see Methods), and release of ^{3}H-ACh evoked by K^{+} was increased up to 251% of control (Fig. 8).

These data indicate that the release of cortical ^{3}H-ACh is, in general, reduced by FN-stimulation. Thus, an FN-elicited release of ^{3}H-ACh, if small in magnitude, would not be detectable.

II. SOURCES OF ACETYLCHOLINE IN CEREBRAL CORTEX

A. Characterization of Microvessels

The MV and synaptosomal fractions were routinely examined by light microscopy to assess their purity and composition. As shown in Figure 9 the preparation contained vessels ranging from 4-40 um in diameter, with minimal contamination by non-vascular elements such as dendrites and glia. The MV fraction was heterogenous in that it contained endothelial and, presumably, smooth muscle cells from arteries and veins (10 - 40 um), and capillaries (< 10 um).

In four separate experiments, the distribution of the diameters of the vascular segments isolated was quantified with a computerized image analysis system. With a joystick-controlled cursor the diameters of the vessels were calculated at the branching points of the vascular tree (Fig. 10A). After counting a total of 690 vessels, 67.7% were less than 10 um (Fig. 10B), which corresponds to a capillary fraction (Nakai et al., 1981). About one third (32.3%) were, therefore, small arterioles and venules ranging from 11 - 40 um (Fig. 10B).

γ-Glutamyltranspeptidase (γ-GTP) and alkaline phosphatase (AP), two marker enzymes which have been localized to intraparenchymal brain vessels (Goldstein et al., 1975; Orlowski et al., 1974; Rowan and Maxwell, 1981; Williams et al., 1980), were enriched in the microvessel fractions both from the CX and CN (Table 4). γ-GTP and AP were enriched by 6 to 29- and 3 to 22-fold, respectively, which is consistent with the values reported by others for fractions containing MVs and capillaries (Goldstein et al., 1975; Estrada et al., 1983).

Further characterization of the MVs at the ultrastructural level demonstrated that many vessels of the CX are surrounded by a thick basement membrane, which encapsulates not only the endothelium but frequently pericytes (Fig. 11A). The smaller blood vessels (4-12 um) were identified as having endothelial cells with a heterochromatic cell nucleus surrounded by a basement membrane and no association with smooth muscle, suggesting that they were capillaries, whereas the larger vessels (> 25 um) had thick basement membranes in association with occasional smooth muscle cells. Both types of blood vessels usually contained intact nuclei, with erythrocytes sometimes found within the lumen of the vessels. Other non-vascular debris such as perikarya was infrequently observed, although material resembling astrocytic end processes and nerve terminals were frequently found attached to the basement membrane. In contrast, the synaptosomal fraction did not contain any vascular elements (Fig. 11B). Nerve terminals and attached postsynaptic elements were found to make both symmetric and asymmetric synapses as previously reported (Gray and Whittaker, 1962; Whittaker, 1969).

ChAT was immunocytochemically localized to axon terminals in the neuropil of layers I, II, III and V (Fig. 12A), as previously described (Houser et al., 1985). These axon terminals contained numerous small clear vesicles (0.1-0.2 um) with peroxidase product surrounding the vesicles. In layer III, small punctate neural elements containing ChAT labeled vesicles were found to come in close apposition to the basement membrane (Fig. 13A) and adjoining pericytes (Fig. 13B) of unlabeled endothelial cells. These elements did not form any classical synaptic or junctional specializations. In other instances, the cytoplasm of capillary endothelial cells were labeled (Fig. 12C and 13). ChAT-immunoreactivity was observed in less than 10% of the endothelial cells. The ChAT labeled endothelial cells usually were associated with small blood vessels (4-8 um) and most frequently observed in layer III of the cerebral cortex. The unlabeled nucleus contained dense clumps of heterochromatin. Thus, a neuroanatomical substrate for the cholinergic innervation of the microvasculature was identified.

B. ChAT Activity in Microvessels

ChAT activity was measured in different fractions isolated from the CX, CN, CRB and LIV. When the data are expressed as the absolute amount of ACh synthesized (pmol ^{14}C-ACh) from each respective fraction and tissue, relative to the amount synthesized from a 100 mg (wet wt.) section of the CX, 2.4% of the total cortical activity was associated with MVs and 94% was associated with synaptosomes (Fig. 14A). No significant differences were observed across the MV fractions from each tissue (Fig. 14A). In contrast, ChAT activity varied 10-fold among the nerve terminal containing fractions (S_1) from the CX, CN, CRB and LIV (Fig. 14A), and in a manner consistent with the known cholinergic innervation to these areas (Fibiger, 1982; Mesulam et al, 1983). However, when the data were calculated as a specific activity (i.e., nmol ^{14}C-ACh formed/mg protein/40 min.), it was discovered that MVs isolated from the three brain structures, but not LIV, had the remarkable capacity to synthesize ^{14}C-ACh at rates significantly greater than the nerve terminal fraction (Fig. 14B). The percent increase above the corresponding homogenate was +95%, +269%, and +313% for the CX, CN and CRB, respectively. These findings suggest that concentrations of ACh synthesized in the brain at the neurovascular junction may be as great, or greater than the transneuronal site.

The ability of cortical MVs to synthesize ACh was confirmed by two additional methods, since ^{14}C-acetyl CoA, which serves as substrate in the assay of ChAT activity, may also serve as substrate for other acetyltransferases present in the brain. First, the specific inhibitor of ChAT, 4-naphthylvinylpyridine (NVP), inhibited ChAT activity in cortical MVs by 95% (Fig. 15). Second, the product formed by incubation of ^{14}C-acetyl CoA with cortical MVs was extracted by the enzymatic liquid-cation exchange method and co-chromatographed with ^{14}C-ACh standards separated by HPLC (Fig. 16). These findings established that elements associated with cortical MVs have the capacity to synthesize authentic ACh.

C. Release of ^{3}H-ACh from Cortical Microvessels

The release of ^{3}H-ACh and, for comparison, endogenous amino acids (AA) neurotransmitters, gamma-aminobutyric acid (GABA), glycine (Gly) and aspartate (Asp) were measured from cortical MVs and nerve terminals (S_1). The pattern of spontaneous release in the presence of 1.2 mM Ca^{2+} is shown in Table 5. For MVs, Gly had the highest and ACh the lowest spontaneous release. In contrast, in the homogenate containing the nerve terminals, GABA had the highest rate of release,

which was 10-fold higher than that observed in the MVs. Also, Gly release was one third less in the homogenate, while release of ACh was 3-fold higher. Asp release was only slightly higher in the homogenate compared to the MVs. The marked differences in the pattern of release suggests that two distinct fractions were isolated and that the release of neurotransmitters from the MV fraction was not the result of a simple contamination from the homogenate.

Cortical MVs depolarized with 55 mM K^+, in the presence of Ca^{2+}, released a substantial amount of ^{3}H-ACh (Fig. 17A). This effect was graded, since depolarization with 25 mM K^+ resulted in smaller, but significant release of ^{3}H-ACh (149 + 19% of the spontaneous release; $p < 0.05$, $N = 5$). Importantly, the K^+-evoked release of ^{3}H-ACh from MVs was entirely dependent on extracellular Ca^{2+} concentrations (Fig. 17B), suggesting a possible neurotransmitter function of the ACh to affect the microvasculature.

Release of GABA, Gly and Asp from MVs by 55 mM K^+ was small and not statistically significant (Fig 17A). In contrast, release of ACh, GABA and Asp, but not Gly, was evoked from the homogenate by 55 mM K^+ (Fig. 17A). Thus cortical MVs may be selectively innervated by cholinergic, but not GABAergic, glycinergic or aspartergic nerves.

DISCUSSION

I. RELEASE OF CORTICAL ACH WITH FN STIMULATION

This study, taken together with previous reports establishing a cholinergic innervation of the cortical cerebral vasculature (Arneric et al., 1986; Duckles, 1982; Eckenstein and Baughman, 1984; Edvinsson et al., 1977; Edvinsson et al., 1972; Estrada et al., 1983; Florence and Bevan, 1979; Gross et al., 1981; Kuschinsky et al., 1974; Lee et al., 1978; Rennels et al., 1977; Vasquez and Purves, 1979), raises the possibility that a subpopulation of cholinergic neurons subserving blood flow regulation releases ACh upon muscarinic cholinergic receptors in the CX to mediate the cortical cerebrovasodilation elicited by electrical stimulation of the fastigial nucleus of the cerebellum. The following evidence supports this conclusion.

Effect of Local Atropine on FN-Elicited Cortical Cerebrovasodilation

We have demonstrated that ATR applied to the right parietal CX attenuates by 59% the cortical vasodilation elicited by FN stimulation in the fronto-parietal CX, while adjacent and contralateral structures are virtually unaffected. The action of ATR to attenuate the FN-elicited response is not the result of vasoparalysis since ATR does not modify the vasodilation elicited by hypercarbia. Nor is it possible that the effect of ATR is a consequence of differences in pCO_2 or pO_2 of arterial blood and superfusate, nor to changes in AP, since these parameters were carefully controlled and did not differ among experimental groups. The specificity of ATR is further supported by the inability of ATR to alter resting rCBF or the cerebrovasodilation elicited by stimulation of the dorsal medullary reticular formation (Iadecola et al., 1983b). The finding that ATR application does not affect resting rCBF indicates it is unlikely that there is any tonic cholinergic activity affecting cortical rCBF. It is also unlikely that the effect of ATR upon the cerebrovascular response to FN-stimulation is mediated by muscarinic receptors located outside the CNS, since this vasodilation occurs through neuronal pathways contained entirely within brain (Nakai et al., 1982; Reis et al., 1985). Thus, the ability of ATR applied locally to the CX to attenuate the FN-elicited increase in cortical rCBF suggests that a substantial component of the cholinergic link resides locally in the CX, and that a population of cholinergic neurons projecting to, or residing within, the CX subserve a phasic role in mediating cortical cerebrovasodilation.

The finding that cortical application of ATR only prevented 59% of the cortical dilation contrasts with the fact that ATR given systemically reduces the vasodilation by 92% (Iadecola et al., 1986b). One explanation for this discrepancy is that in the present study the area of the parietal CX removed for analysis is larger than the area affected by application of ATR. Thus contamination by cortex not affected by ATR, in which vasodilation occurred to a maximal extent, would lead to an underestimation of the ability of ATR to block the vasodilation. Another possibility is that a co-transmitter may be required for the dilation to occur. Although less likely, this possibility would be consistent with the recent report that ACh and vasoactive intestinal polypeptide another potent vasodilator of cerebral vessels (Heistad et al., 1980), co-exist in a subpopulation of local cortical neurons (Eckenstein and Baughman, 1984). Additional experiments using local cortical microinjection of ATR and autoradiographic techniques to measure local cortical blood flow will be required to resolve these possibilities.

The ability of local ATR application to block the FN-elicited increase in rCBF could be related to the release of ACh onto cerebral vessels and/or upon neurons. Muscarinic receptors are localized to all segments of the cerebral vasculature, including large cerebral arteries (Estrada and Krause, 1982), small pial arteries (Estrada et al., 1983; Estrada and Krause, 1982) and capillaries (Estrada et al., 1983; Estrada and Krause, 1982), wherein receptors are present on both endothelial and/or smooth muscle cells. Muscarinic receptors localized to cortical neurons have a heterogeneous distribution, with highest concentrations corresponding to high levels of ChAT activity (Johnston et al., 1979; Kuhar and Yamamura, 1976; Wamsley et al., 1984) (mostly laminae III-IV). Interestingly, the greatest increases in cortical rCBF elicited by FN-stimulation are also found in laminae III-IV (Nakai et al., 1983). On the basis of our study, we cannot ascertain whether the effect of ATR is due to blockade of vascular and/or neuronal muscarinic receptors

A neurogenic vasodilator mechanism originating in the brainstem, ascending to the CX and involving one or more cholinergic links within brain which release ACh in the vicinity of cortical blood vessels has been postulated by Scremin and his associates (Hudson et al., 1985; Scremin et al., 1973; Scremin et al., 1978; Scremin et al., 1983). The stimulus for the cortical vasodilation and release of ACh they report is hypercarbia. However, in apparent contradiction with their hypothesis, we did not demonstrate an effect of ATR locally applied to the CX to attenuate the cortical cerebrovasodilation produced by hypercarbia. Although the reasons for the differences between the experiments are unclear at the present time, both laboratories still support the concept of a neural regulation of the cortical cerebrovasculature that is phasic and cholinergic.

Cortical Release of ACh Following FN-Stimulation

The fact that a substantial portion of the cortical vasodilation is mediated via local muscarinic cholinergic receptors, suggests that ACh must be released in the CX and participate in the vasodilation. However, we were unable to detect the expected stimulus-locked release of ACh during FN-stimulation. This lack of effect is not the result of our inability to accurately measure the release of ACh from the CX, since potassium depolarization resulted in a marked stimulus-locked release of ACh. In our experiments potassium depolarization elevated the release of ACh by 151%, which is of a comparable magnitude to the maximally evoked release of ACh reported by others for rats and cats (50-200%; Pepeu, 1973; Yaksh and Yamamura, 1975). In addition, in 5 of 5 experiments in which rCBF and release of ACh was measured concurrently, cortical rCBF was elevated to the expected levels by FN stimulation. Thus, local vasoparalysis or an impaired reactivity of the tissue as a result of the ^{3}H-choline microinjection could not account for our inability to detect increased ACh release.

The failure to detect release of ACh from CX with FN stimulation might indicate that endogenous ACh is contained in a different releasable pool than is the pool labeled by microinjection of ^{3}H-choline. However, this possibility is unlikely for two reasons. First, Yaksh and Yamamura (1975) have shown in cats that neuronal pools containing endogenous ACh and those containing ACh newly synthesized from radiolabeled choline, if not identical, are released in the same manner following depolarization of the nerve terminals. Second, in three additional experiments that were performed to avoid the possible problems associated with using radiolabeled choline, we could not detect an increase in the release of endogenous ACh following FN-stimulation as measured by gas chromatographic/mass spectrophotometric techniques (Arneric at al., 1986b).

Taken together, we interpret these results to suggest that during FN-stimulation the amount of ACh released by the subpopulation of cholinergic neurons producing local vasodilation is too small to be detected by currently available methods.

It is possible that the small but significant decrease in the release of ACh during FN-stimulation is related to a more generalized decrease in cholinergic activity not directly involved with cerebrovascular regulation. Others have demonstrated that there may be a direct relationship between the amount of ACh released from the cortical surface and the level of generalized brain activity as indicated by the cortical EEG (Jasper and Tessier, 1971; Pepeu, 1973; Szerb, 1967). In addition, stimulation of the FN does not substantially alter cortical cerebral glucose utilization (Nakai et al., 1983) and, in fact, slightly decreases the frequency of the cortical EEG (Underwood et al., 1983; and present study). Thus, in addition to the presumed evoked release of ACh involved with the cortical cerebrovasodilation, FN-stimulation may act through other, perhaps parallel, neuronal pathways to modify release of ACh not related to cerebral blood flow regulation.

The source of ACh released within the CX following FN stimulation and the neural pathway from FN to CX is unknown. Neuroanatomical tracing studies show that projections from FN do not reach the CX directly (Del Bo et al., 1982). Therefore, the pathway from FN to the CX must be polysynaptic. The two most likely sources for the cholinergic link in this pathway are local cholinergic neurons of the cortex, or nerve terminals of afferent cholinergic fibers arising from or passing through the basal forebrain (Eckenstein and Baughman, 1984; Johnston et al., 1979; Lehman et al., 1980; Mesulam et al., 1983). While lesions of the basal forebrain (Iadecola, 1983a) both reduce cortical ChAT activity and prevent the cortical vasodilation elicited by FN stimulation, these experiments still do not establish whether the source of the ACh is in afferent or local neurons. Recent experiments using cortical microinjection of ibotenic acid, an excitotoxin that causes restricted perikaryal lesions without damaging fibers of passage (Iadecola et al., 1986a) have demonstrated that local cortical neurons are obligatory to mediate the increase in rCBF produced by FN-stimulation. The results of the present study indicate that these intrinsic cortical neurons may be either cholinergic, or receive a cholinergic input. Although the origin of the cellular elements releasing ACh in the cerebral cortex during FN stimulation is unknown, this study supports the possibility that a local cortical cholinergic mechanism plays a significant role in mediating the vasodilation elicited from the FN.

II. SOURCES OF ACH IN CEREBRAL CORTEX

This study demonstrates that elements associated with small (< 40 um) intraparenchymal blood vessels in the rat CX, including endothelial cells and cholinergic nerve terminals closely apposed to the basal lamina, have the capacity to synthesize, store and release ACh following depolarization with K^+. Previous physiological and pharmacological experiments demonstrate that ACh is released from large cerebral vessels (Duckles, 1981) and that these vessels dilate in response to ACh (Duckles, 1981; Florence and Bevan, 1979; Lee et al., 1978). Thus, it is possible that the synthesis and release of ACh, at the level of cortical intraparenchymal vessels, may also provide a potent mechanism for the neural control of the cerebral circulation.

Cholinergic Innervation of Cerebral Microvessels

Although the present study confirms earlier reports that MVs isolated from

rat and bovine CX contain ChAT activity (Estrada et al., 1983; Goldstein et al., 1975; Santos-Benito and Gonzales, 1985), it differs from those reports in the quantity (i.e. activity) of ChAT found there. Previous reports indicated that intraparenchymal vessels from the rat CX have 2-5% of the specific activity associated with cortical grey matter (Table 6) (Goldstein et al., 1975; Santos-Benito and Gonzales, 1985). Our study also indicates cortical MVs constitute a small fraction (2.3%) of the total ACh biosynthetic capacity of the CX. However, in addition it reveals that based on specific activities, MVs isolated from the CX, CN and CRB have the remarkable ability to synthesize ^{14}C-ACh at rates 100-300% greater than the nerve terminal fraction (Fig. 12B). This raises the possibility that the concentrations of ACh synthesized at the cortical neurovascular junction may be as great, or greater than the transneuronal site.

There are two reasons that could explain the differences between the ChAT activities measured in this and previous studies. First and foremost, the distribution of the size of the vessel diameters isolated were different, which could result in a differential sampling of those vessels innervated by cholinergic nerves. Earlier reports (Goldstein et al., 1975; Santos Benito and Gonzales, 1985) indicate that 90-95% of the vessels were < 10 um in diameter and, therefore, capillaries. This study demonstrates that only two thirds of the vessels sampled were capillaries, while the remaining one third consisted of vessels 11 - 40 um in diameter. Second, the isolation procedure used in the earlier reports are lengthier and may have resulted in a deterioration in the viability of the ChAT activity, although this is less likely because the values for the other marker enzymes were similar between the studies. Together, these data suggest that unlike capillaries in the CX, small precapillary arterioles, or perhaps postcapillary venules, have a substantial capacity to synthesize ACh.

Additional data indicate that the ChAT activity measured in the MV fraction is in direct association with the microvasculature and not the result of contamination from cortical gray matter. First, ultrastructural examination of sections of CX immunocytochemically stained for ChAT revealed that capillary endothelial cells and nerve terminals that directly contact the basal lamina of MVs actually contain ChAT (Fig. 13A and B). Second, light and electron microscopic level examination of the MV fractions suggest that perikaryal contamination was minimal. Third, the enriched activities of -GTP and AP suggest that distinct fractions were isolated. Fourth, the cerebellum, which is known to contain relatively low levels of gray matter ChAT activity (Estrada, et al., 1983), had in the MV fraction an equivalent capacity to the CX to synthesize ACh (Fig. 14A). Also, the cerebellar MVs have a higher specific activity of ChAT than the tissue of origin (Fig. 14B). If contamination had occurred, it would be expected to be similar for all regions since the isolation procedure used was the same. The enhanced specific activities associated with the MV fraction and the unpredicted ratios of MV to gray matter activities of ChAT argue against contamination as a significant factor in determining the activities measured. Finally, preliminary studies indicate that even when the whole S_1 fraction (which contains 95% of the total cortical ChAT activity) is deliberately layered over the discontinuous sucrose gradient to contaminate the preparation less than 50% of the activity normally observed from the P_1 fraction is measured. Therefore, in the worst possible case, the activity measured is approximately 2-fold greater than the actual value. This lower value for the specific activity of ChAT for cortical MVs is still at least 10-fold greater than any previous report (Table 6).

The concept of an innervation of capillaries and other segments of the microvasculature is not new (Rennels et al., 1977). However, this study is the first to demonstrate that depolarization of isolated intraparenchymal blood vessels results in the Ca^{2+}-dependent release of a putative neurotransmitter such as ACh. As shown by the ultrastructural studies, there are two possible sources of the released ACh found in association with the MVs. Both endothelial cells (Fig. 13C and D) and nerve terminals (Fig. 13A) that closely appose pericytes or the basement membrane contain ChAT. Further experimentation is required to determine which of these cellular source(s) of ChAT contributed to the synthesis and K^+-evoked release of ^{3}H-ACh.

Other types of neurotransmitters may also innervate the microvasculature. Anatomical and physiological evidence suggest noradrenergic fibers originating in the locus coeruleus innervate capillaries (Hartman et al., 1980). Peptide transmitters such as substance P and neurotensin have been shown at the ultrastructural level, using immunocytochemical techniques, to directly innervate capillaries (Milner et al., 1986a; 1986b). These same transmitters, like ACh (Hamel et al., 1985), have been shown to innervate larger cerebral vessels (Itakura et al., 1984). However, the release data from this study suggests that some cortical neurotransmitters do not innervate the microvasculature.

GABA, a neurotransmitter in the CX whose source arises almost exclusively from local neurons (Emson et al., 1979), is an example of a transmitter that may not innervate the microvasculature. GABA and aspartate (Fonnum et al., 1981), another amino acid transmitter found in the CX, is not released from the MV fraction, while release of both amino acids occurs from the nerve terminal fraction (Fig. 17A). This inability of GABA to be released from MVs contrasts with the biochemical evidence that GABAergic neurons innervate pial arteries (Krause et al., 1980; Hamel et al., 1982), and that these vessels dilate in response to GABA agonists (Edvinsson and Krause, 1979). Together these reports support the idea that each segment of the vasculature is differentially innervated. Moreover, the pattern of spontaneous release that occurs in either the MV or the nerve terminal fraction further supports the contention that the MV fraction was not systematically contaminated by gray matter.

Functional Implications of Microvessel Innervation

The function of neurotransmitters that innervate cortical intraparenchymal vessels is unclear, since this level of the microvasculature (i.e. < 50 um) is relatively devoid of smooth muscle cells with which transmitters could interact with to cause vasodilation (Edvinsson et al., 1981). Despite this paradox, there is now good evidence that cholinergic neurotransmission exists at this level of the vasculature, since ChAT activity, acetylcholinesterase activity, ACh release and muscarinic receptors are all found in association with capillaries and endothelial cells (Edvinsson et al., 1972, 1977; Estrada et al., 1983; Estrada and Krause, 1982; Goldstein et al., 1975; Parnavalas et al., 1985; present study). Moreover, ChAT, generally considered a specific marker of cholinergic neurons (Mesulam et al., 1983), is found both in nerve terminals that closely appose the basement membrane and in non-neuronal endothelial cells (Parnavalas et al., 1985; present study). This type of innervation appears non-classical in that synaptic specializations are absent. This has, perhaps, led others to suggest that cholinergic transmission may be important in modulating less anatomically restricted functions such as amino acid transport (Duckles, 1982), or capillary permeability (Parnavalas et al., 1985).

There are, however, two other possible mechanisms by which release of ACh could still regulate local tissue perfusion. Although not yet confirmed, cholinergic nerve terminals may innervate precapillary sphincters that divert local cerebral blood flow (Nakai et al., 1981). Alternatively, endothelial cells and adjoining pericytes could modulate the diameter of the MVs directly since these cells contain contractile proteins such as actin (Owman et al., 1984). This possibility seems plausible, since decreasing ACh synthesis in sperm, another non-neuronal cell that contains ChAT (Sastry and Sadavongvivad, 1979), results in decreased cell motility.

Anatomical Organization of Cortical Cholinergic Systems: Relationships to Function

The origin and anatomical organization of the cortical cholinergic systems may have important implications for the functions of ACh in the CX. It is presumed that fibers innervating cerebral capillaries would be of intracerebral, rather than peripheral, origin since there is no perivascular space at this vascular level (Rennels et al., 1978). If this is so, then there are four independent sources of ACh within brain which could influence the cerebral vasculature. The major source (80%) of cholinergic innervation to the CX arises from underlying neurons in the basal forebrain (Fibiger and Lehmann, 1981; Fibiger, 1982; Mesulam et al., 1983). Another less dense (5% of the total activity) extrinsic projection arises from neurons in the dorsal mesencephalic reticular formation (Mufroniew et al., 1985). Intrinsic cholinergic neurons with cell bodies and nerve terminals present in the CX have been confirmed by several investigators (Eckenstein and Thoenen, 1983; Houser et al., 1985; Wainer et al., 1984) and are thought to comprise 10-15% of the cortical innervation. Lastly, capillary endothelial cells contain less than 2% of the total ACh biosynthetic capacity of the CX (present study).

The functions of cholinergic fibers in the CX is undoubtedly dependent upon the type of neurons or neuroeffector sites which they innervate. Light microscopic examination of the cortical cholinergic fiber system shows a loosely organized network throughout all cortical layers with many of the terminal boutons associated with distal dendrites rather than with more proximal portions of cortical neurons (Houser et al., 1985). This pattern is distinctly different from the cortical GABAergic system, for example, in which axon terminals concentrate around perikarya to create the appearance of specific associations with identifiable neurons (Houser et al., 1983). The apparently diffuse nature of the fibers and terminals is consistent with a global, modulatory role for the cholinergic system in the CX. This contention is supported by physiological evidence that ACh can enhance the response of cortical neurons to other transmitters as well as to other inputs (Krnjevic, 1981). Physiological studies also report that there is a laminar difference in the response of cortical neurons to ACh (Lamour et al., 1982), with neurons in layer V being especially responsive. These findings are consistent with the enhanced density of cholinergic fibers found in layers I-III and V of somatic-sensory cortex (Houser et al., 1985). However other areas of CX show a more subtle variance in the laminar pattern (Houser et al., 1985) Finally, in addition to the specificity of action imparted by anatomical innervation, pharmacological studies indicate that further diversity in function and physiological action (i.e. excitation versus inhibition) can be imparted by different subtypes of cholinergic receptors (Marchi and Raiteri, 1985).

The fact that the cholinergic fibers in the CX arise both from intrinsic and extrinsic sources supports the possibility that ACh plays more than one role in cortical function. It suggests that the different sources of cholinergic innervation may each serve a different role in maintaining cortical brain function. In fact, one

of these systems may be dedicated to cerebral blood flow regulation. However, the exact role that each cholinergic system participates in remains to be elucidated.

An example of a possible functional dichotomy between intrinsic and extrinsic cholinergic neurons is supported by the finding that intrinsic cholinergic neurons also contain vasoactive intestinal polypeptide (VIP), a potent cerebral vasodilator (Edvinsson et al., 1981; Heistad et al., 1980), whereas extrinsic neurons in the basal forebrain do not (Eckenstein and Baughman, 1984). It has been suggested that neurons containing ACh and VIP may be involved in the regulation of cortical blood flow (Eckenstein and Baughman, 1984), since peripheral neurons containing both these substances synergistically increase blood flow in exocrine glands (Lundberg, 1981). Electrophysiological studies are consistent with this suggestion for they indicate VIP can enhance the response of cortical neurons to ACh simultaneously applied (Lamour et al., 1983). In addition, VIP has been shown to enhance ChAT activity in some brain regions (Luine et al., 1984). Thus, intrinsic cortical neurons containing ACh and VIP may be devoted to modulating the cerebral vasculature.

Although a proximity of some ChAT containing elements have been mentioned previously (Eckenstein and Baughman, 1984), no synaptic or junctional specializations between these elements and blood vessels are documented. This report is the first to demonstrate at the ultrastructural level that cholinergic elements, perhaps nerve terminals, contact the basement membrane of the cortical microvasculature, seemingly without any other intended synaptic associations. The origin of these cholinergic elements, and whether or not they also contain VIP is not known. However, it reinforces the concept that the cholinergic innervation to MVs is without classical synaptic or junctional specializations. More importantly, it provides the neuroanatomical substrate for cholinergic control of the cortical microvasculature.

The nature of the physiological stimulus that activates the intrinsic cholinergic systems involved in cerebral blood flow regulation is unknown. However, this laboratory has discovered a neural pathway in brain that, when electrically stimulated, increases blood flow in brain globally (Nakai et al., 1980, 1983). This global cerebrovasodilation is totally abolished by systemic administration of the muscarinic cholinergic receptor antagonist, atropine (Iadecola et al., 1986), indicating that release of ACh is involved in mediating the response. Recent studies indicate that application of atropine to a restricted region of the CX attenuates the response locally (Arneric et al., 1986). This cerebrovasodilator pathway must be phasically active since blood flow is unaffected by atropine in unstimulated animals. Moreover, selective lesions of the CX with ibotenic acid, an excitotoxin that destroys perikarya without affecting fibers of passage (Schwartz et al., 1979; Iadecola et al., 1986), also totally abolished the evoked cerebrovasodilation within the lesion, without affecting adjacent regions (Iadecola et al., 1986). Taken together, these data support the idea that the terminals from local cholinergic neurons in the CX are one subpopulation of the cholinergic innervation of the CX involved in controlling cerebral microcirculation.

REFERENCES

Amaral, D.G. and Price, J.L. (1983) An air pressure system for the injection of tracer substances into the brain. J. Neurochem. 19 35-44.

Armstrong, D.M. (1986) Ultrastructural characterization of choline acetyltransferase-containing neurons in the basal forebrain of rat: evidence for a cholinergic innervation of intracerebral blood vessels. J. Comp. Neurol. 250, 81-92.

Arneric, S.P., Iadecola, C., Honig, M.A., Underwood, M.D. and Reis, D.J. (1986a) Local cholinergic mechanisms mediate cerebral cortical vasodilation elicited by electrical stimulation of the fastigial nucleus. Acta Physiol. Scand., in press.

Arneric, S.P., Iadecola, C., Underwood, M.D. and Reis, D.J. (1986b) Local cholinergic mechanisms participate in the increase in cortical cerebral blood flow elicited by electrical stimulation of the fastigial nucleus in rat. Brain Res. (submitted).

Arneric, S.P., Meeley, M.P. and Reis, D.J. (1986c) Somatostatin and CCK-8 modulate release of striatal amino acids: role of dopamine receptors. Peptides 7, 97-103.

Arneric, S.P. and Reis, D.J. (1986) Somatostatin and cholecystokinin octapeptide differentially modulate the release of ^{3}H-acetylcholine from caudate nucleus but not cerebral cortex: role of dopamine receptor activation, Brain Res. 374, 153-161.

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Bresolin, N., Freddo, L., Vergani, L. and Angelini, C. (1982) Carnitine, carnitine acyltransferases, and rat brain function. Exp. Neurol. 78, 285-292.

Briggs, C.A. and Cooper, J.A. (1981) A synaptosomal preparation from the guinea pig ileum myenteric plexus. J. Neurochem. 36, 1097-1108.

Del Bo, A., Ruggiero, D.A., Ross, C.A., Wiley, R. and Reis, D.J. (1982) Pathways from the fastigial pressor area in rat. Soc. Neurosci. Abstr. 8, 76.

Duckles, S.P. (1981) Evidence for a functional cholinergic innervation for cerebral arteries. J. Pharmacol. Exp. Therap. 217, 544-548.

Duckles, S.P. (1982) Choline acetyltransferase in cerebral arteries: modulator of amino acid uptake? J. Pharmacol. Exp. Therap. 223, 716-720.

Eckenstein, F. and Baughman, R.W. (1984) Two types of cholinergic innervation in cortex, one co-localized with vasoactive intestinal polypeptide. Nature 309, 153-155.

Eckenstein, F. and Thoenen, H. (1983) Cholinergic neurons in the rat cerebral cortex demonstrated by immunocytochemical localization of choline acetyltransferase. Neurosci. Lett. 36, 211-215.

Edvinsson, L. Falck, B. and Owman, C. (1977) Possibilities for a cholinergic action on smooth musculature and on sympathetic axons in brain vessels mediated by muscarinic and nicotinic receptors. J. Pharmacol. Exp. Ther. 200, 117-126.

Edvinsson, L. and Krause, D.N. (1979) Pharmacological characterization of GABA receptors mediating vasodilation of cerebral arteries in vitro. Brain Res. 173, 89-97.

Edvinsson, L., Nielsen, K.C., Owman, C. and Sporrong, B. (1972) Cholinergic mechanisms in pial vessels. Histochemistry, electron microscopy and pharmacology, Z. Zellforsch., 134, 311-325.

Estrada, C., Hamel, E. and Krause, D.N. (1983) Biochemical evidence for cholinergic innervation of intracerebral blood vessels. Brain Res. 266, 261-270.

Estrada, C. and Krause, D. (1982) Muscarinic cholinergic receptor sites in cerebral blood vessels. J. Pharmacol. Exp. Ther. 221, 85-90.

Florence, V.M. and Bevan, J. (1979) Biochemical determination of cholinergic innervation of cerebral arteries. Circ. Res. 45, 212-218.

Furchgott, R.F., Zawadzki, J.V. and Cuerry, P.D. (1981) Role of endothelium in the vasodilator response to aceylcholine, In: Vasodilation (P.M. Vanhoutte and I. Leusen, eds.) Raven Press, New York, pp. 49-66.

Goldstein, G.W., Wolinsky, J.S., Csejtey, J. and Diamond, I. (1975) Isolation of metabolically active capillaries from rat brain. J. Neurochem. 25, 715-717.

Grammas, P., Diglio, C.A., Marks, B.H., Giacomelli, F. and Wiener, J. (1983) Identification of muscarinic receptors in rat cerebral corticial microvessels. J. Neurochem. 40, 645-651.

Gray, E.G. and Whittaker, V.P. (1962) The isolation of nerve endings from brain: an electron microscopic study of cell fragments derived from homogenization and centrifugation. J. Anatomy 96, 79-88.

Gross, P.M., Harper, A.M. and Graham, D.I. (1981) Cerebral blood flow in rats during physiological and humoral stimuli. Stroke 12, 345-352.

Hadhazy, P. and Szerb, J.C. (1977) The effect of cholinergic drugs on ^{3}H-acetylcholine release from slices of rat hippocampus, striatum and cortex. Brain Res. 123, 311-322.

Hamel, E., Krause, D.N. and Roberts, E. (1982) Characterization of glutamic acid decarboxylase activity in cerebral blood vessels. J. Neurochem. 39, 842-849.

Harik, S.I., Sharma, Y.K., Wetherbee, J.R., Warren, R.H. and Banerjee, S.P. (1981) Adrenergic and cholinergic receptors or cerebral microvessels. J. Cerebr. Blood Flow Metab. 1, 329-338.

Hartman, B.K., Swanson, L.W., Raichle, M.E., Preskorn, S.H. and Clark, H.B. (1980) Central adrenergic regulation of cerebral microvascular permeability and blood flow; anatomic and physiological evidence. Advanc. Exp. Med. Biol. 131, 113-126.

Heistad, D.D., Marcus, M.L., Said, S.I. and Gross, P.M. (1980) Effect of acetylcholine and vasoactive intestinal polypeptide on cerebral blood flow. Am. J. Physiol. 239, H73-H80.

Hernandez, M.J., Brennan, R.W. and Bowman, G.S. (1978) Cerebral blood flow autoregulation in the rat. Stroke 9, 150-155.

Hösli, E. and Hösli, L. (1982) Evidence for the existence of alpha- and beta-adrenoceptors on neurones and glial cells of cultured rat central nervous system - an autoradiographic study. Neuroscience 7(11), 2873-2881.

Houser, C.R., Crawford, G.D., Salvaterra, P.M. and Vaugn, J.E. (1985) Immunocytochemical localization of choline acetyltransferase in rat cerebral cortex: a study of cholinergic neurons and synapses. J. Comp. Neurol. 23, 17-34.

Houser, C.R., Hendry, S.H.C., Jones, E.G. and Vaughn, J.E. (1983) Morphological diversity of immunocytochemically identified GABA neurons in the monkey sensory-motor cortex. J. Neurocytol. 12, 617-638.

Hudson, D.M., Jensen, D.J., Scremin, O.U. and Sonneschein, R.R. (1985) Cortical acetylcholine efflux with hypercapnia and nociceptive stimulation. Brain Res. 338, 267-272.

Iadecola, C., Arneric, S.P., Baker, H.D., Tucker, L.W. and Reis, D.J. (1986a) Role of local neurons in the cerebrocortical vasodilation elicited from the cerebellum. Am. J. Physiol. (Submitted).

Iadecola, C., Mraovitch, S., Meeley, M.P. and Reis, D.J. (1983a) Lesions of the basal forebrain in rat selectively impair the cortical vasodilation elicited from cerebellar fastigial nucleus. Brain Res. 279, 41-52.

Iadecola, C., Underwood, M., Ishitsuka, T. and Reis, D.J. (1983b) Role of the cholinergic system in the cerebrovascular vasodilation elicited by stimulation of the dorsal medullary reticular formation in rat. Neurosci. Abst. 9, 773.

Iadecola, C., Underwood, M.D. and Reis, D.J. (1986b) Muscarinic cholinergic receptors mediate the cerebrovasodilation elicited by stimulation of the cerebellar fastigial nucleus in rat. Brain Res. 368, 375-379.

Itakuro, T., Okuno, T., Nakakita, K., Kamei, I., Naka, Y., Nakai, K., Imai, H., Komai, N., Kimura, H. and Maeda, T. (1984) A light and electron microscopic immunohistochemical study of vasoactive intestinal polypeptide- and substance P-containing nerve fibers along the cerebral blood vessels: comparison with aminergic and cholinergic fibers. J. Cerebral Blood Flow Metab. 4, 407-414.

Jasper, H.H. and Koyama, I. (1969) Rate of release of amino acids from the cerebral cortex in the cat as affected by brainstem and thalamic stimulation. Can J. Physiol. Pharmacol. 47, 889-905.

Jasper, H.H. and Tessier, J. (1971) Acetylcholine liberation from cerebral cortex during paradoxical (REM) sleep. Science 172, 601-602.

Johnston, M.V., McKinney, M. and Coyle, J.T. (1979) Evidence for a cholinergic projection to neocortex from neurons in basal forebrain. Proc. Natl. Acad. Sci. 76, 5392-5396.

Kety, S.S. (1951) The theory and applications of the exchange of inert gas in the lungs and tissues. Pharmacol. Rev. 3, 1-41.

Krause, D.N., Roberts, E., Wong, E., Degener, P. and Rogers, K. (1980) Specific cerebrovascular localization of GABA-related receptors and enzymes, Brain Res. Bull. 5(2), 173-177.

Krnjevic, K. (1981) Transmitters in motor systems. In: The Nervous System, Vol. II, Motor Control (V.B. Brooks, ed.) Baltimore, American Physiological Society, pp. 107-154.

Kuhar, M.J. and Yamamura, H.I. (1976) Localization of cholinergic muscarinic receptors in rat brain by light microscopic radioautography. Brain Res. 110, 229-243.

Kuschinsky, W., Wahl, M. and Neiss, A. (1974) Evidence for cholinergic dilatory receptors in pial arteries of cats. Pflugers Arch. 347, 199-208.

Lamour, Y., Dutar, P. and Jobert, A. (1982) Excitatory effect of acetylcholine on different types of neurons in the first somatosensory neocortex of the rat: laminar distribution and pharmacological characteristics. Neuroscience 7, 1483-1494.

Lamour, Y., Dutar, P. and Jobert, A. (1983) Effects of neuropeptides on rat cortical neurons: laminar distribution and interaction with the effect of acetycholine. Neuroscience 10(1), 107-117.

Lee, T.J.F., Hume, W.R., Su, C. and Bevan, J.A. (1978) Neurogenic vasodilation of cat cerebral arteries. Circ. Res. 42, 535-542.

Lehman, J., Nagy, J.I., Atmadja, S. and Fibiger, H.C. (1980) The nucleus basalis magnocellularis: the origin of a cholinergic projection to the neocortex of the rat. Neuroscience 5, 1161-1174.

Lundberg, J.M. (1981) Evidence for coexistence of vasoactive intestinal polypeptide (VIP) and acetylcholine in neurons of cat exocrine glands. Morphological, biochemical and functional studies. Acta Physiol. Scand. (Suppl.) 496, 1-57.

Marchi, M. and Raiteri, M. (1985) On the presence in the cerebral cortex of muscarinic receptor subtypes which differ in neuronal localization. function and pharmacological properties. J. Pharmacol. Exp. Therap. 235(1), 230-233.

Mesulam, M.M., Mutson, E.J., Wainer, B.H. and Levey, A.I. (1983) Central cholinergic pathways in the rat: an overview based on an alternative nomenclature (Ch.1 - Ch.6), Neuroscience 10, 1185-1201.

McCaman, R.E., McCaman, M.W. and Stafford, M.L. (1966) Carnitine acetyltransferase in nervous tissue. J. Biol. Chem. 241(4), 930-934.

Miura, M. and Reis, D.J. (1969) Cerebellum: A pressor response elicited from the fastigial nucleus and its efferent pathway in brainstem. Brain Res. 13, 595-599.

Moruzzi, G. and Magoun, H.W. (1949) Brainstem reticular formation and activation of the EEG. Electroenceph. clin. Neurophysiol. 1, 455-473.

Nakai, K., Imai, H., Kamei, I. Itakura, T., Komari, N., Kimura, H., Nagai, T. and Maeda, T. (1981) Microangioarchitecture of rat parietal cortex wth special reference to vascular "sphincters". Stroke 12(5), 653-659.

Nakai, M., Iadecola, C. and Reis, D.J. (1982) Global cerebral vasodilation elicited by stimulation of rat fastigial cerebellar nucleus. Am. J. Physiol. (Heart & Circ. Physiol.) 243, H226-H235.

Nakai, M., Iadecola, C., Ruggiero, D.A., Tucker, L.W. and Reis, D.J. (1983) Electrical stimulation of cerebellar fastigial nucleus increases cerebral cortical blood flow without change in local metabolism: Evidence for an intrinsic system in brain for primary vasodilation. Brain Res. 260, 35-49.

Nathan, M.A. and Reis, D.J. (1975) Hypoxemia, atelectasis, and elevation of arterial pressure and heart rate in paralyzed artificially ventilated rat. Life Sci. 16, 1103-1120.

Ohno, K., Pettigrew, K.D. and Rappoport, S.I. (1979) Local cerebral blood flow in the conscious rat as measured with ^{14}C-iodoantipyrine and ^{3}H-nicotine. Stroke 10, 62-67.

Orlowski, M. and Meister, A. (1965) Isolation of γ-glutamyl transpeptidase from hog kidney. J. Biol. Chem. 240, 338-347.

Orlowski, M., Sessa, G. and Green, J.P. (1974) Gamma-glutamyltranspeptidase in brain capillaries: possible site of a blood-brain barrier for aino acids. Science 184, 66-68.

Oldendorf, W.H. (1971) Brain uptake of radiolabelled amino acids, amines and hexoses after arterial injection. Am. J. Physiol. 221(6), 1629-1639.

Osborne, N.N. (1981) Communication between neurons: current concepts. Neurochem. Int. 3(1), 3-16.

Palkovits, M. (1984) Distribution of neuropeptides in the central nervous system: a review of biochemical mapping studies. Prog. Neurobiol. 23, 151-189.

Parnavelas, J.C., Kelly, W. and Burnstock, G. (1985) Ultrastructural localization of choline acetyltransferase in vascular endothelial cells in rat brain. Nature 316, 724-725.

Pepeu, G. (1973) The release of acetylcholine from the brain: an approach to the study of the central cholinergic mechanisms. Prog. Neurobiol. 3, 259-288.

Reinhard, J.F., Liebmann, J.E., Schlossberg, A.J. and Moskowitz, M.A. (1979) Serotonin neurons project to small blood vessels in brain. Science 106, 85-87.

Reis, D.J., Iadecola, C., MacKenzie, E., Mori, M., Nakai, M. and Tucker, L. (1982) Primary and metabolically coupled cerebrovascular dilation elicited by stimulation of two intrinsic systems in brain. In D. Heistad and M. Marcus (Eds.), Cerebral Blood Flow: Effects of Nerves and Neurotransmitters, Elsevier/North Holland, New York, pp. 475-484.

Reis, D.J., Iadecola, C. and Nakai, M. (1985) Control of cerebral blood flow and metabolism by intrinsic neural systems in brain. In: F. Plum and W. Pulsinelli, (Eds.), Cerebrovascular Diseases, Raven Press, New York, pp. 1-25.

Rennels, M.L., Forbes, M.S., Anders, J.J. and Nelson, E. (1977) Innervation of the microcirculation in the central nervous system and other tissues. In: C. Owman and L. Edvinsson, (Eds.), Neurogenic Control of Brain Circulation, Pergammon Press, Oxford, pp. 91-104.

Richter, J.A. and Marchbanks, R.M. (1971) Synthesis of radioactive acetylcholine from (^{3}H)-choline and its release from cerebral cortex slices in vitro. J. Neurochem. 18, 691-703.

Rowan, R.A. and Maxwell, D.S. (1981) An ultrastructural study of vascular proliferation and vascular alkaline phosphatase activity in the developing cerebral cortex of the rat. Am. J. Anat. 160, 257-265.

Sakurada, O., Kennedy, C., Jehle, J., Brown, J.D., Carbin, G.L. and Sokoloff, L. (1978) Measurement of local cerebral blood flow with iodo (^{14}C) antipyrine. Am. J. Physiol. 234 (Heart and Circ. Physiol.), H59-H66.

Santos-Benito, F.F. and Gonzales, J.L. (1985) Decrease of choline acetyltransferase activity of rat cortex capillaries with aging. J. Neurochem. 45, 633-636.

Sastry, B.V.R. and Sadavongvivad, C. (1979) Cholinergic systems in nonnervous tissues. Pharmacol. Rev. 30, 65-132.

Scremin, O.U., Rovere, A.A., Raynald, A.C. and Giardini, A. (1973) Cholinergic control of blood flow in the cerebral cortex of the rat. Stroke 4, 232-239.

Scremin, O.U., Rubinstein, E.H. and Sonnenschein, R.R. (1978) Cerebrovascular CO_2 reactivity: role of a cholinergic mechanism modulated by anesthesia. Stroke, 9 160-165.

Scremin, O.U., Sonnenshcein, R.R. and Rubenstein, E.H. (1983) Cholinergic cerebral vasodilation: lack of involvement of cranial parasympathetic nerves. J. Cereb. Blood Flow Metab. 3, 362-368.

Szerb, J.C. (1967) Cortical acetylcholine release and electroencephalographic arousal. J. Physiol. (Lond.) 192, 329-343.

Tucek, S., Havranek, M. and Ge, I. (1978) Synthesis of (acetyl-^{14}C)carnitine and the use of tetraphenylboron for differential extraction of (acetyl-^{14}C)choline and (acetyl-^{14}C)carnitine. Anal. Biochem. 84, 589-593.

Underwood, M.D., Iadecola, C. and Reis, D.J. (1983) Stimulation of brain areas increasing cerebral cortical blood flow and/or metabolism does not activate the electroencephalogram. J. Cereb. Blood Flow Metab. 3(Suppl. 1), S218-219.

Vasquez, J. and Purves, M.J. (1979) The cholinergic pathways to cerebral blood vessels. I. Morphological studies. Pflugers Arch. 379, 157-163.

Walz, W. and Hertz, L. (1983) Functional interactions between neurons and astrocytes. II. Potassium homeostasis at the cellular level. Prog. Neurobiol. 20, 133-183.

Wamsley, J.K., Zarbin, M.A. and Kuhar, M.J. (1984) Distribution of muscarinic cholinergic high and low affinity agonist binding sites: a light microscopic autoradiographic study. Brain Res. Bull. 12, 233-243.

Whittaker, V.P. (1969) The synaptosome. In: Handbook of Neurochemistry, Vol. 2 (A. Lajtha, ed.), Plenum Press, New York, pp. 327-364.

Williams, S.K., Gillis, J.F., Matthews, M.A., Wagner, R.C. and Bitensky, M.W. (1980) Isolation and characterization of brain endothelial cells: morphology and enzyme activity. J. Neurochem. 35, 374-381.

Yaksh, T.L. and Yamamura, H.I. (1975) The release in vivo of (^{3}H)-acetylcholine from cat caudate nucleus and cerebral cortex by atropine, pentylenetetrazol, K^+depolarization and electrical stimulation. J. Neurochem. 25, 123-130.

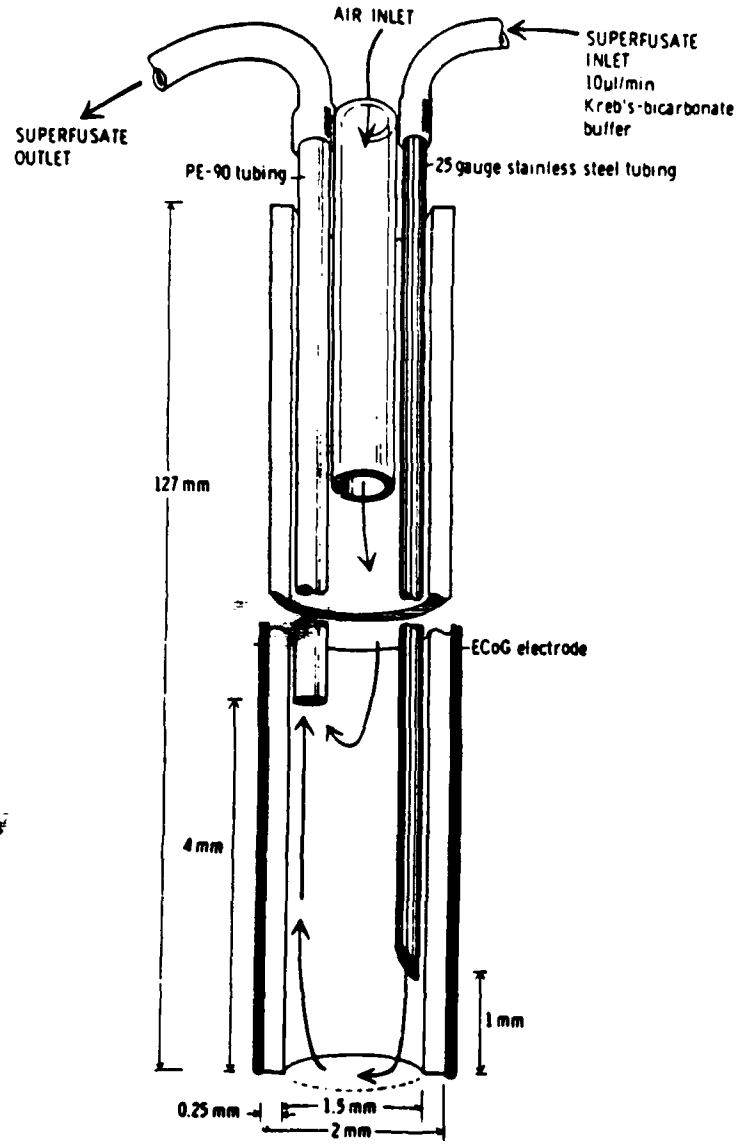


Fig. 1A. A schematic of the device used to apply atropine to the cortical surface and collect superfusate during the release experiments.

Fig. 1B. The region of the parietal cortex sampled during these experiments is shown following introduction of fast green dye into the superfusion device to mark the placement.

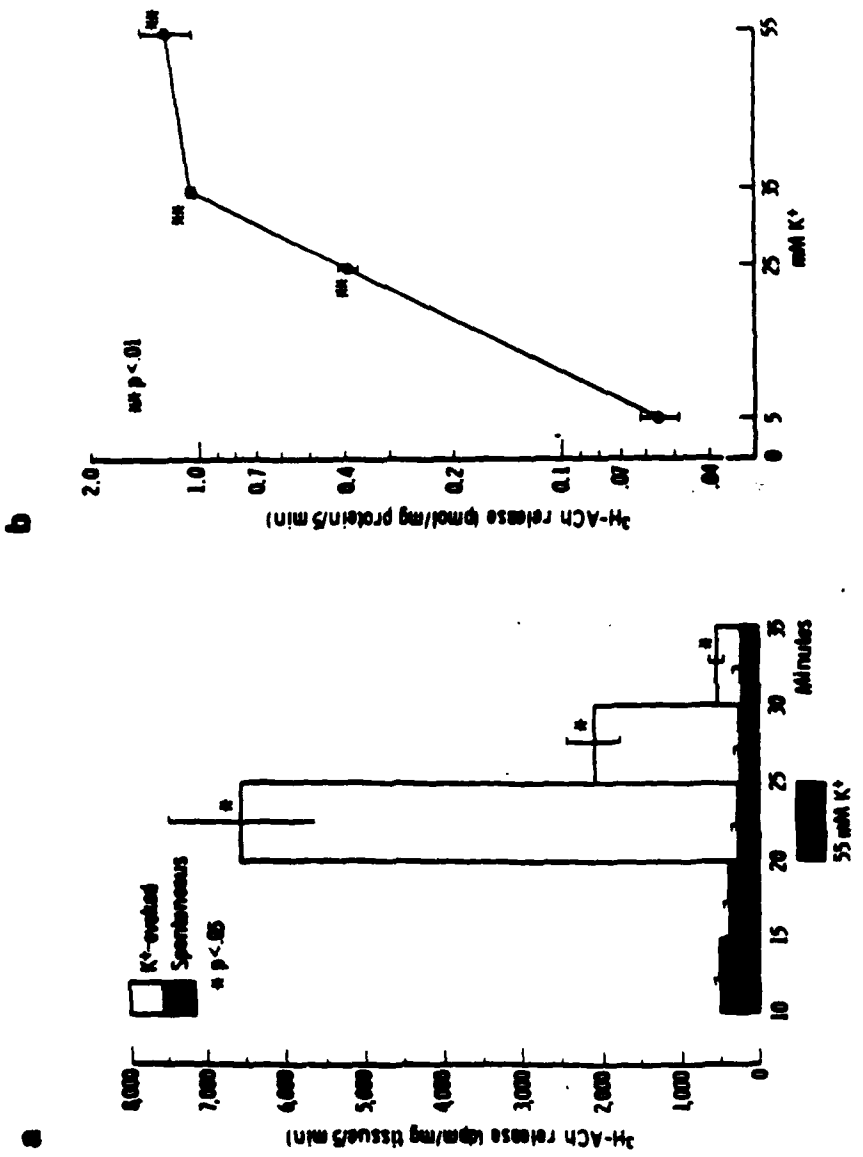


Fig. 2. Release of ^{3}H-ACh from tissue slices of the targeted area of CX during steady-state spontaneous efflux and following depolarization with 55 mM K^{+} (panel A). Panel B shows the effect of varying the potassium depolarization stimulus. Values are means ± S.E.M. (N=3).

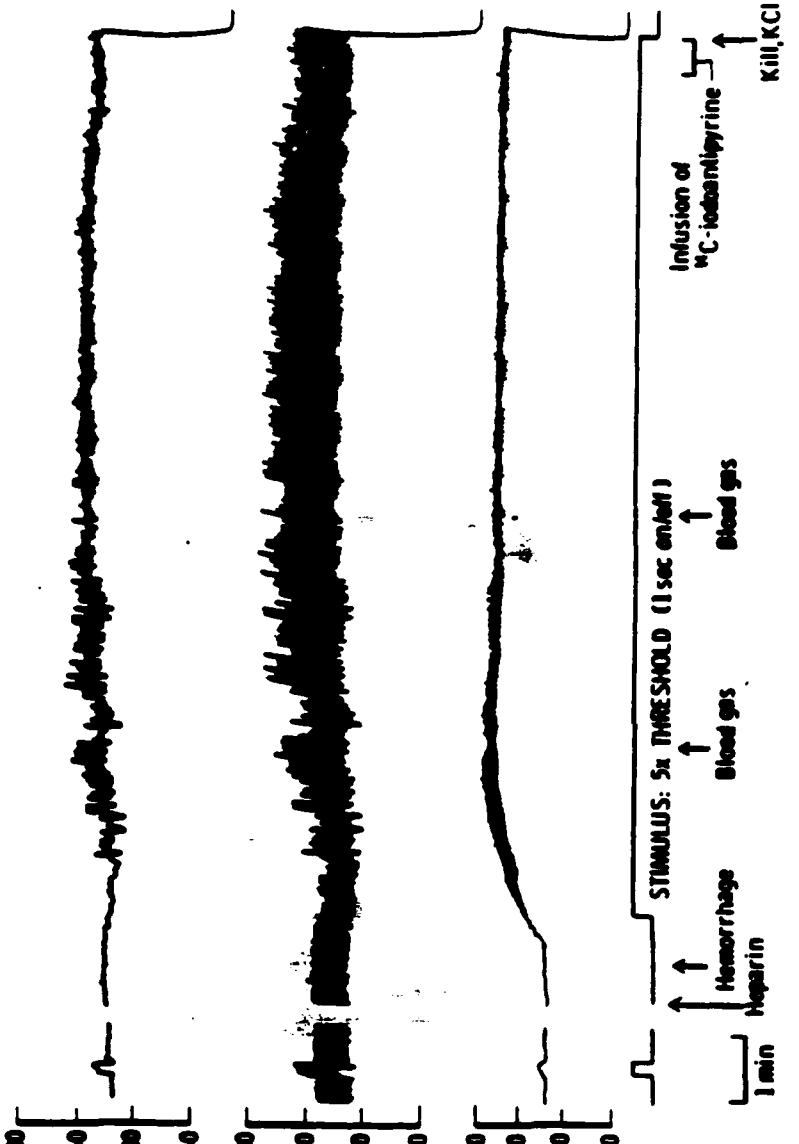


Fig. 3. An illustration of the later portion of a representative blood flow experiment summarizing the protocol used to measure rCBF during electrical stimulation of the fastigial nucleus (FN) of the cerebellum. Three hours prior the animal was anesthetized with choloralose, paralyzed with d-tubocurarine and ventilated with 100%. Note that during FN stimulation mean AP is maintained within the autoregulated range (80-150 mmHg), and that arterial blood gases are monitored and adjusted.

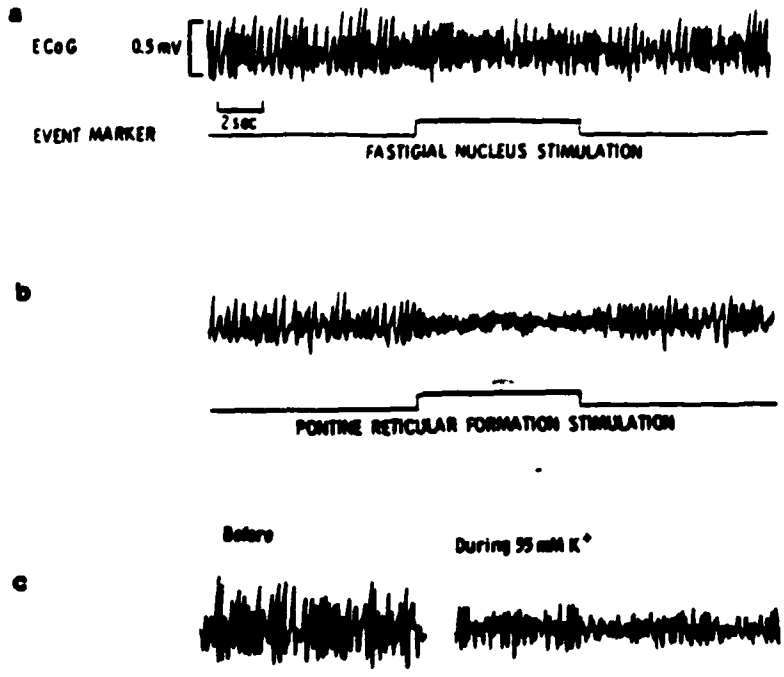


Fig. 4. Representative tracings of the ECoG recorded from the cerebral cortex underlying the superfusion device following electrical and chemical stimulation of different brain regions. Note the difference in the ECoG following electrical stimulation (100 uA, 50 Hz, 0.3 msec) of the fastigial nucleus (panel A) or the pontine reticular formation (panel B). Importantly, the classical cortical "arousal response" described by Moruzzi and Magoun (1949) can be elicited while the superfusion device is in place on the cortical surface. Panel C shows the electrocorticogram (ECoG) immediately before and two minutes after initiating depolarization of the parietal Cx with local application of K^+ (55 mM).

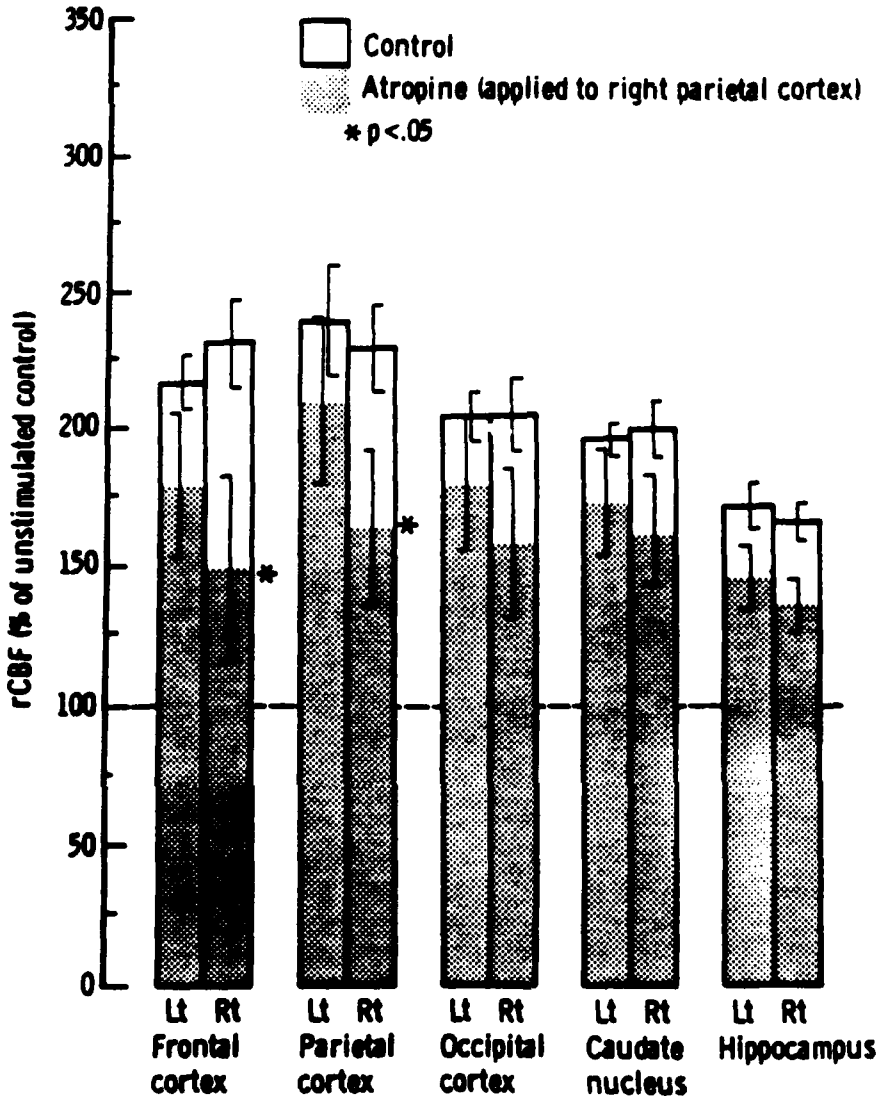


Fig. 5. Effect of atropine sulfate (100 uM) applied to the right parietal cortex (vehicle to left) on the increases in rCBF elicited by electrical stimulation of the FN. FN-stimulation increased rCBF symmetrically (paired t-test, right to left; $p > 0.05$) and significantly (ANOVA, $p < 0.01$, $N = 11$) as compared to unstimulated controls ($N = 6$). Note that atropine applied 20 min prior to rCBF measurement attenuated by 59% the FN-elicited increase in rCBF in the fronto-parietal CX region without affecting rCBF in other adjacent areas ($N = 5$, $p < 0.05$). Values are means $\pm$ S.E.M.

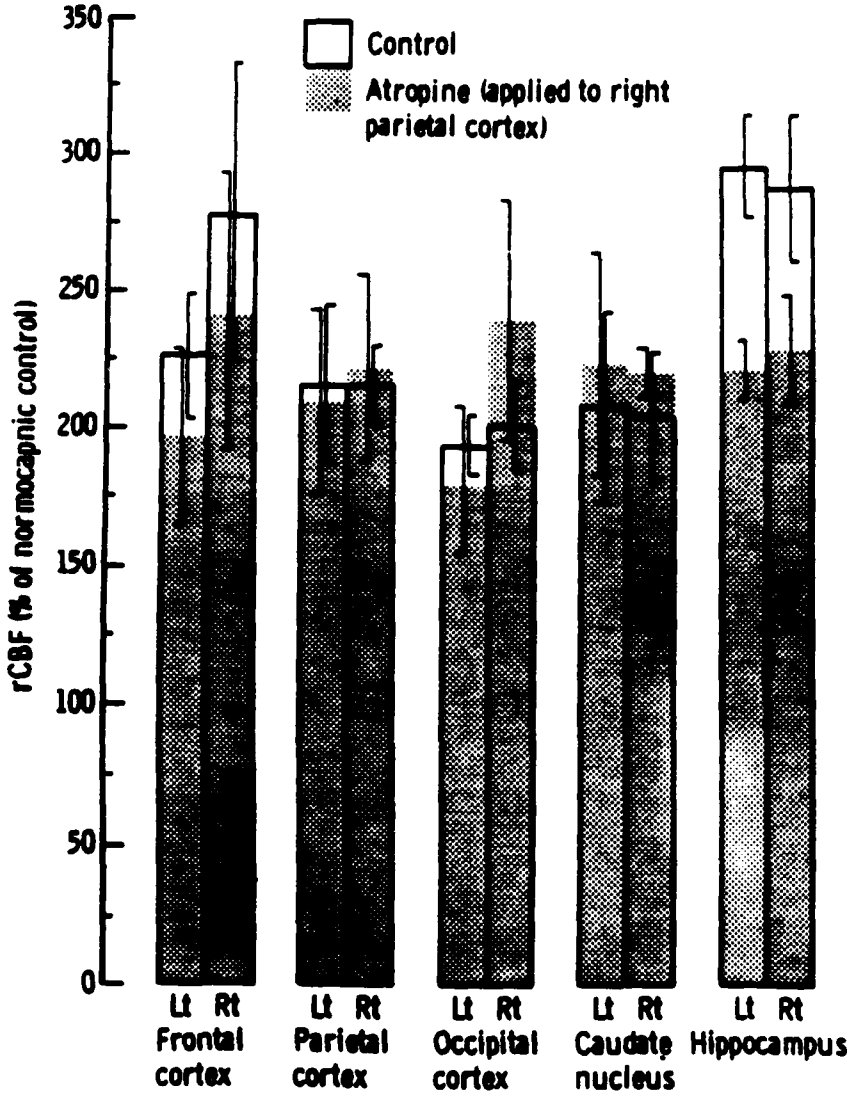


Fig. 6. Effect of atropine sulfate (100 uM) applied to the right parietal cortex on CO_2-elicited increases in rCBF. Arterial pCO_2 was elevated with 5% CO_2 inhalation to 59.0 ± 1.4 mmHg in 5 rats, which increased rCBF to a similar level obtained by FN-stimulation. However, atropine applied 20 min prior to rCBF measurement did not significantly affect the cortical cerebrovasodilation produced by hypercarbia ($p > 0.05$). Values are means ± S.E.M.

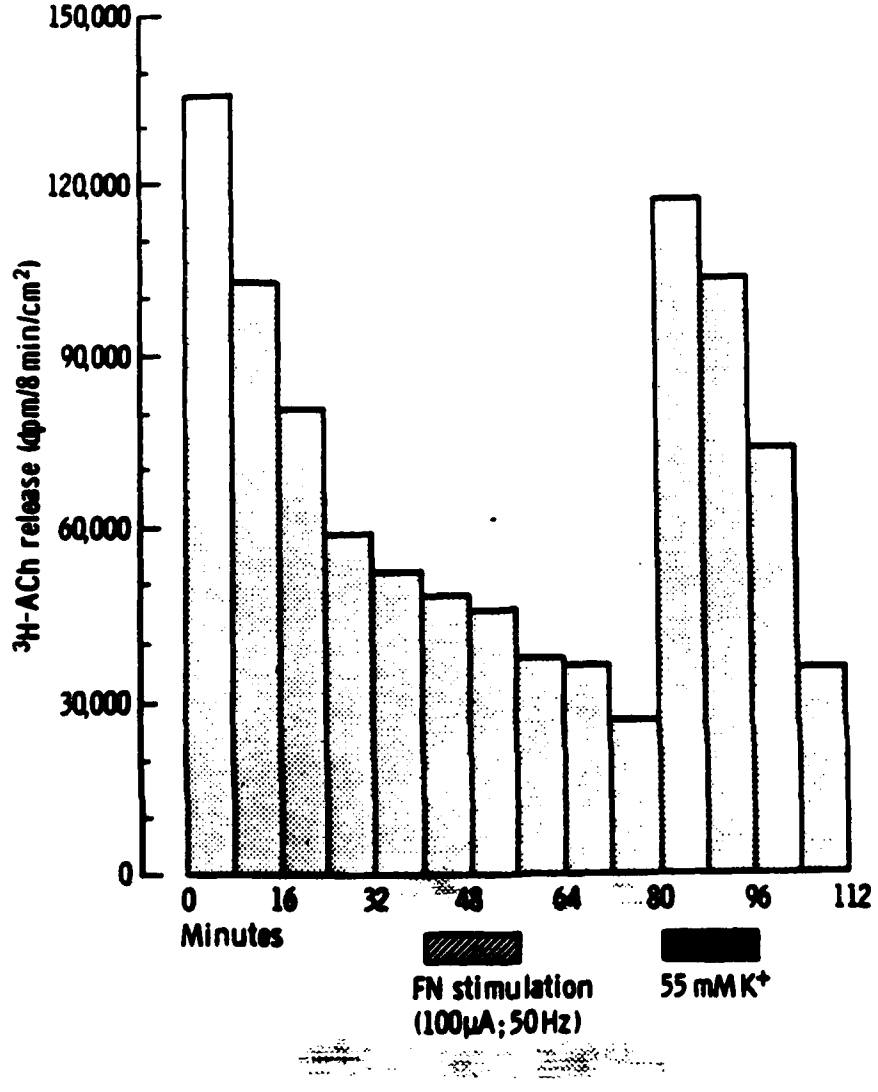


Fig. 7. A representative experiment showing the effect of electrical stimulation of the fastigial nucleus or potassium depolarization on the release of ^{3}H-ACh from the parietal Cx of anesthetized, paralyzed and artificially ventilated rats.

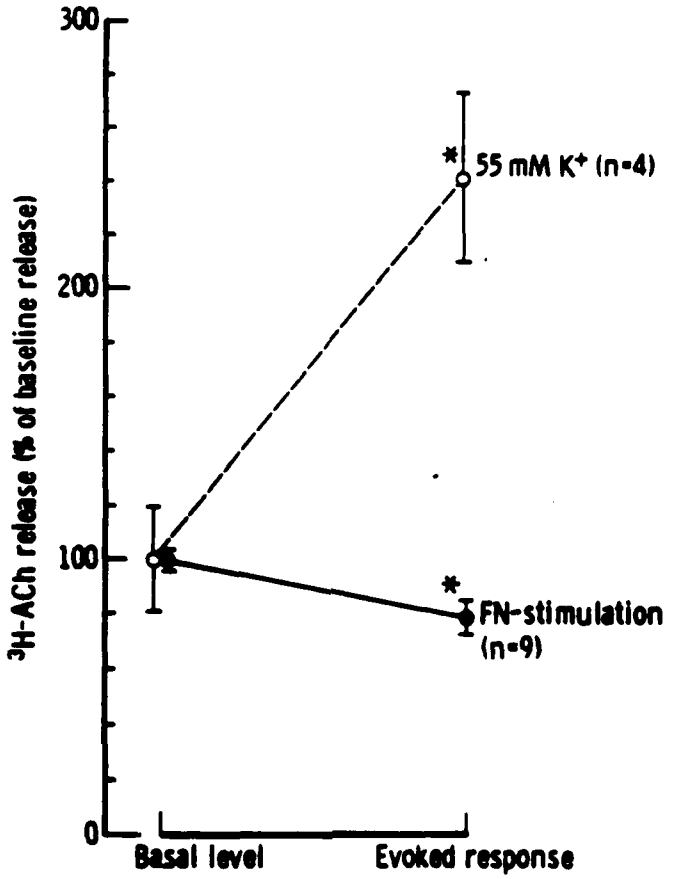


Fig. 8. Grouped data demonstrating the effect of potassium depolarization (55 mM) or electrical stimulation of the fastigial nucleus (FN) on the release of ^{3}H-ACh from the parietal Cx of anesthetized (chloralose), paralyzed and artificially ventilated rats. Values are means + S.E.M.; * indicates the value is significantly decreased from basal control values ($p < 0.05$).

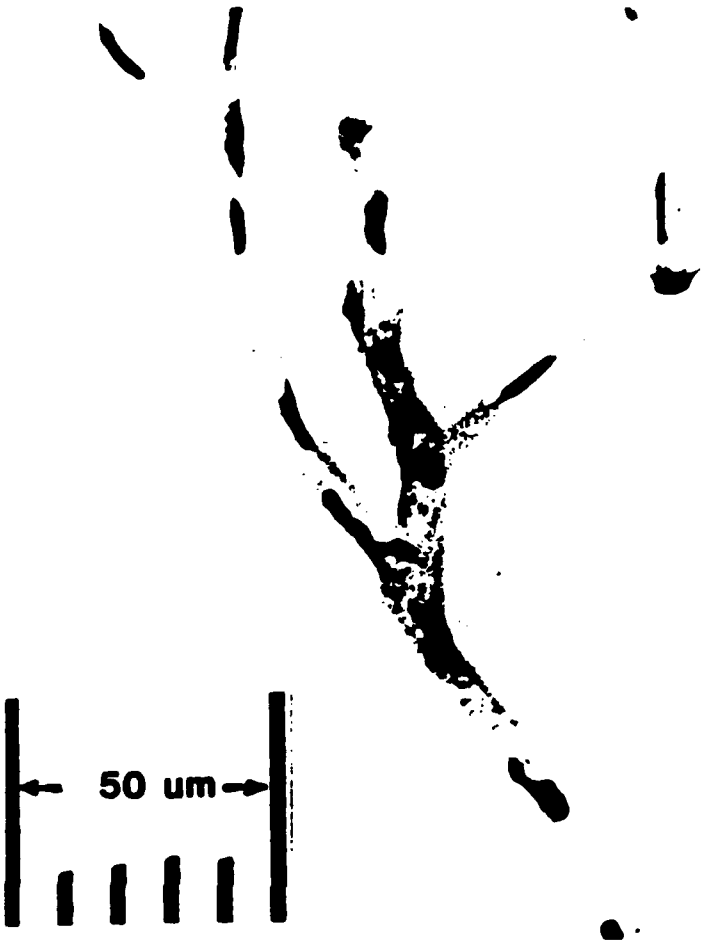


Fig. 9. Light level photomicrograph of microvessels prepared from the cerebral cortex and stained with methylene blue.

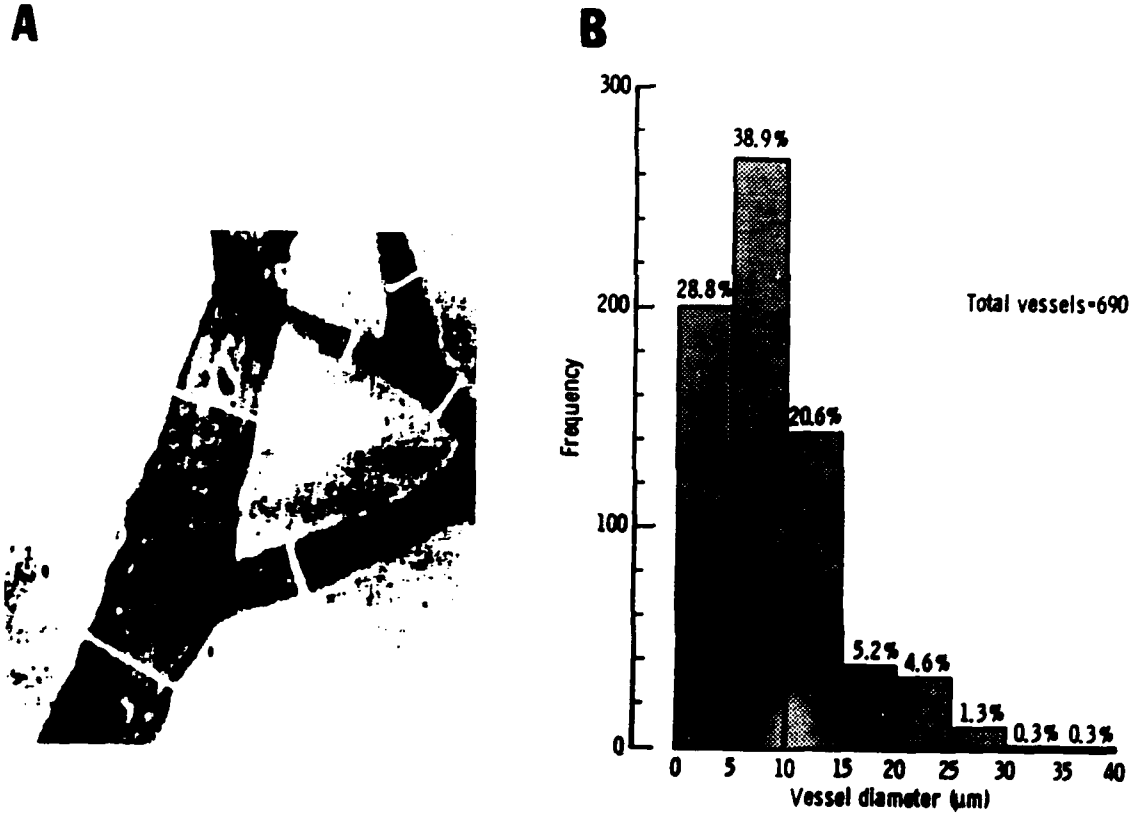


Fig. 10. Panel A—A light level photomicrograph of the cortical microvessel preparation as viewed from the video screen. With the aid of a joystick-controlled cursor and a computerized image analysis system, the diameters of the vessels were calculated at the branching points of the vascular tree (see arrows). Panel B--The distribution of the diameters of 690 vessels isolated from four separate experiments is represented.

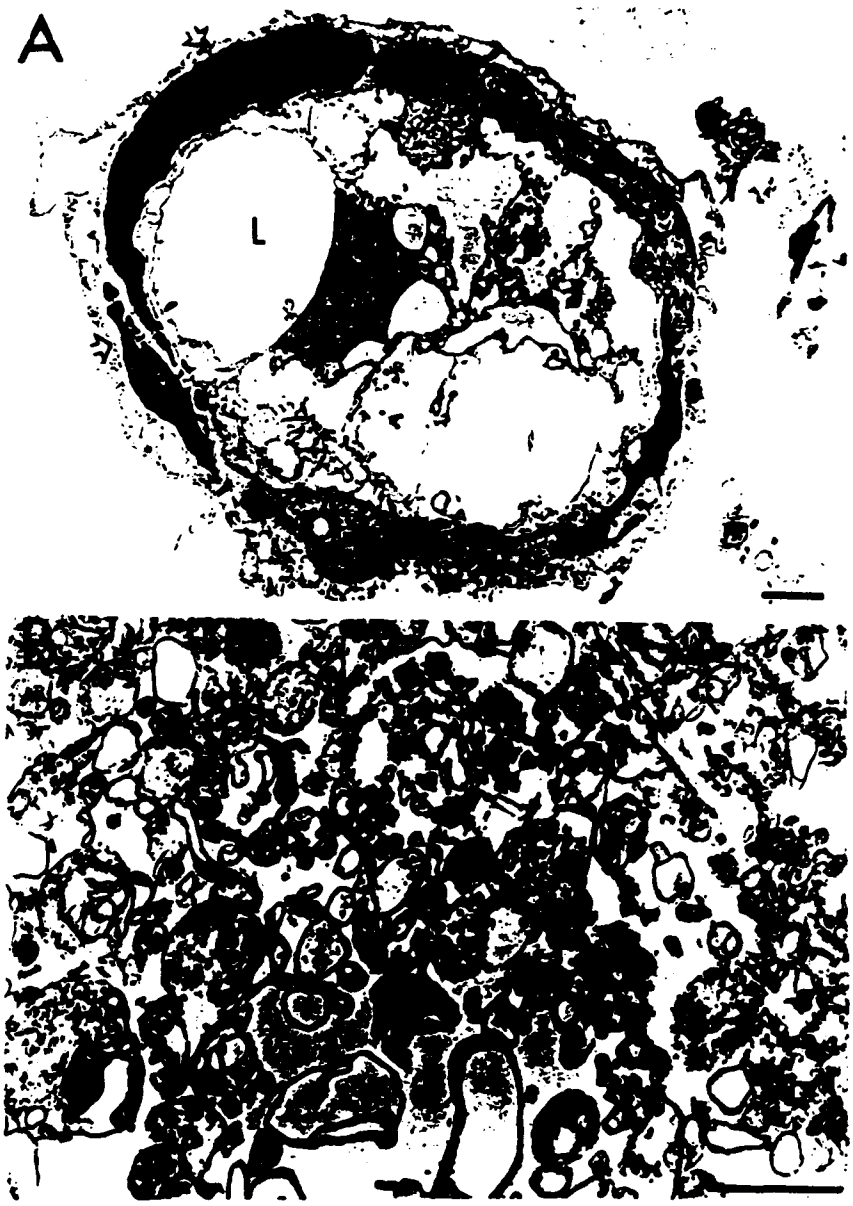


Fig. 11. Panel A—An electron photomicrograph of a representative microvessel isolated from the cerebral cortex detailing the ultrastructural morphology of the vessels examined in these studies. Bar = 1 um. Panel B—An electron photomicrograph of the synaptosomal preparation of the cerebral cortex. Bar = 0.25 um.

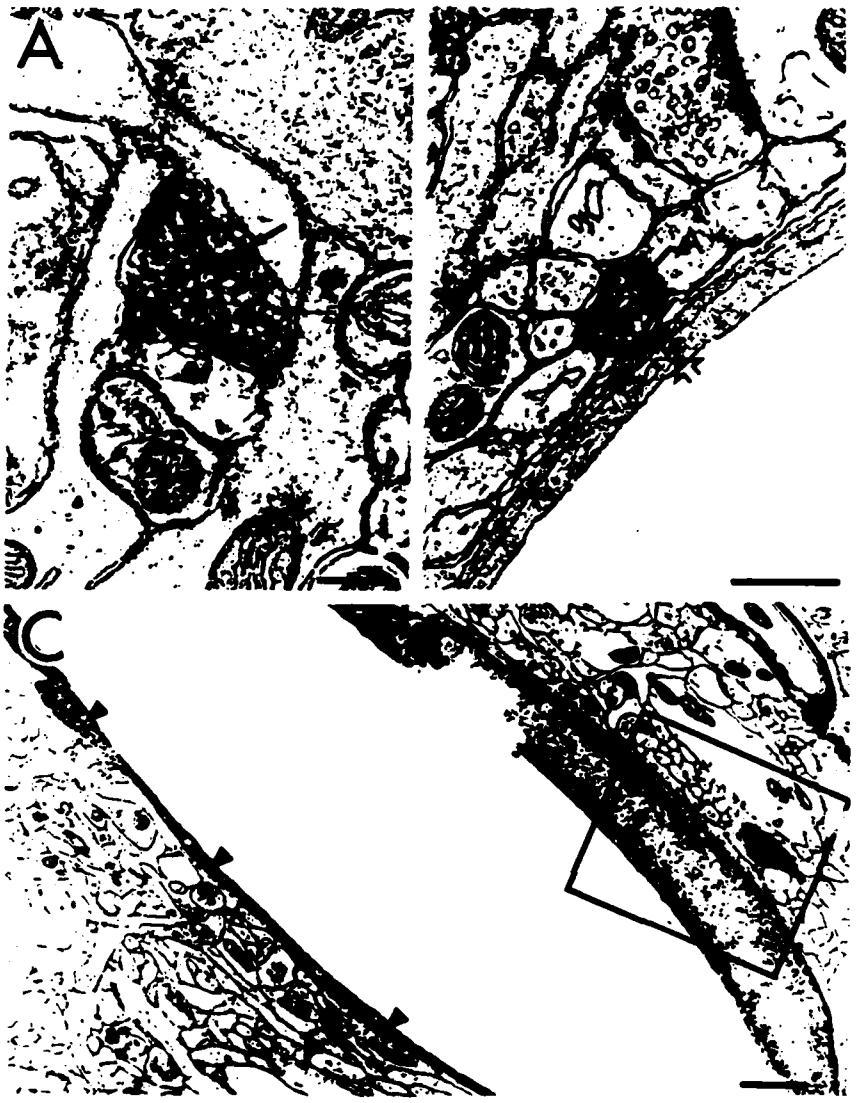


Fig. 12. Panel A—An electron photomicrograph of the neuropil in the cerebral cortex showing the presence of a ChAT containing axon terminal. The section was stained immunocytochemically with a monoclonal antibody to ChAT (Eckenstein and Thoenen, 1983; Boehringer-Mannheim Biochemicals) and localized in the tissue by a modification (Pickel, 1981) of the peroxidase anti-peroxidase (PAP) technique of Sternberger (1979). Bar = 0.25 um. Panel B—A labeled ChAt-stained axon (A) is adjacent to the basement membrane of an unlabeled blood vessel and is separated by a thin astrocytic process (*). Bar = 0.25 um. Panel C—An endothelial cell (En) lining the blood vessel is more intense than that of the labeled terminal. The boxed area containing a ChAT-stained terminal is enlarged in Figure 13 . Bar = 0.5 um.

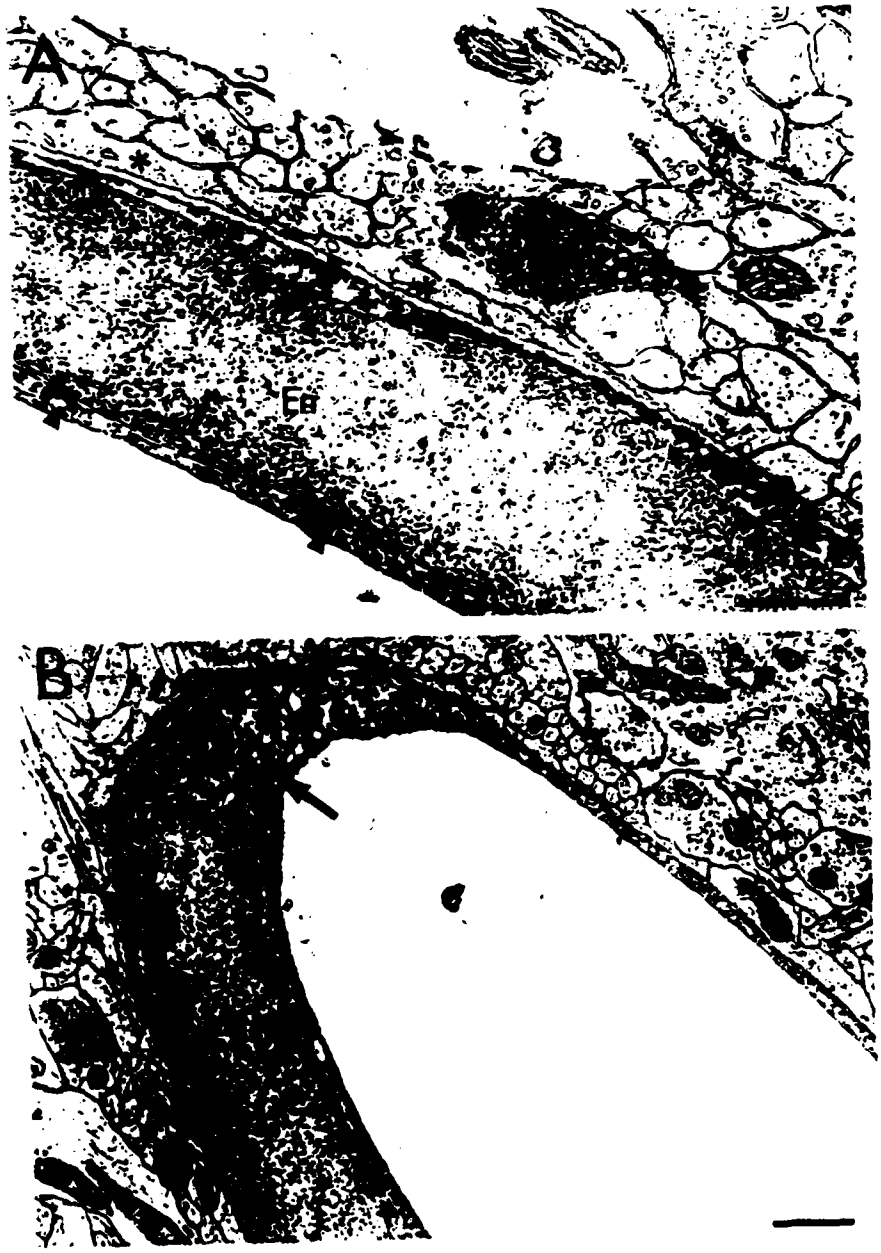


Fig. 13. Photomicrographs of the rat cerebral cortex demonstrating the specific ultrastructural localization of elements in layer III of cerebral cortex immunocytochemically stained for ChAT (Eckenstein and Thoenen, 1983; Boehringer-Mannheim Biochemicals). Panel A—Higher magnification of the boxed area in Fig. 12 shows that the labeled terminal (T) which contains numerous small clear vesicles (SCVs) is separated from the ChAT-labeled endothelial cell (arrowheads) by a thin astrocytic process (*). Bar = 0.25 um. Panel B—A serial section of the endothelial cell in panel A showing the dense PAP product in the cytoplasm (arrow). Bar = 0.25 um.

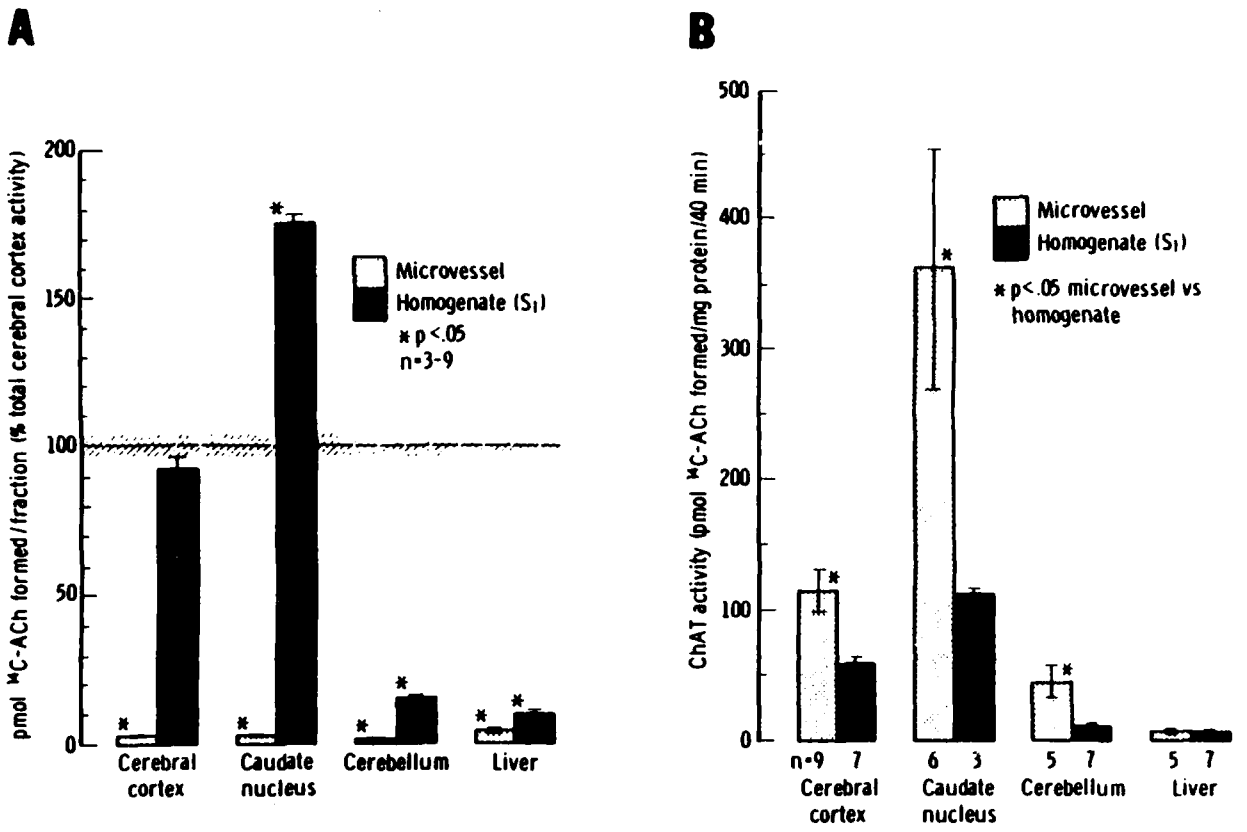


Fig. 14. Panel A—ChAT activity measured in different tissues and subcellular fractions when expressed as the absolute amount of ^{14}C-ACh synthesized relative to the amount synthesized from a 100 mg (wet wt.) section of the cerebral cortex. Values are means ± S.E.M.; * indicates the value is significantly different from the cerebral cortex whole homogenate value, $p < 0.05$. Panel B—The same data as in Panel A, except now it represents ChAT activity from different tissues and subcellular fractions expressed as a specific activity (i.e. nmol ^{14}C-ACh formed/mg protein/40 min). Values are means ± S.E.M.; * indicates the value is significantly increased above the nerve terminal fraction, $p < 0.05$.

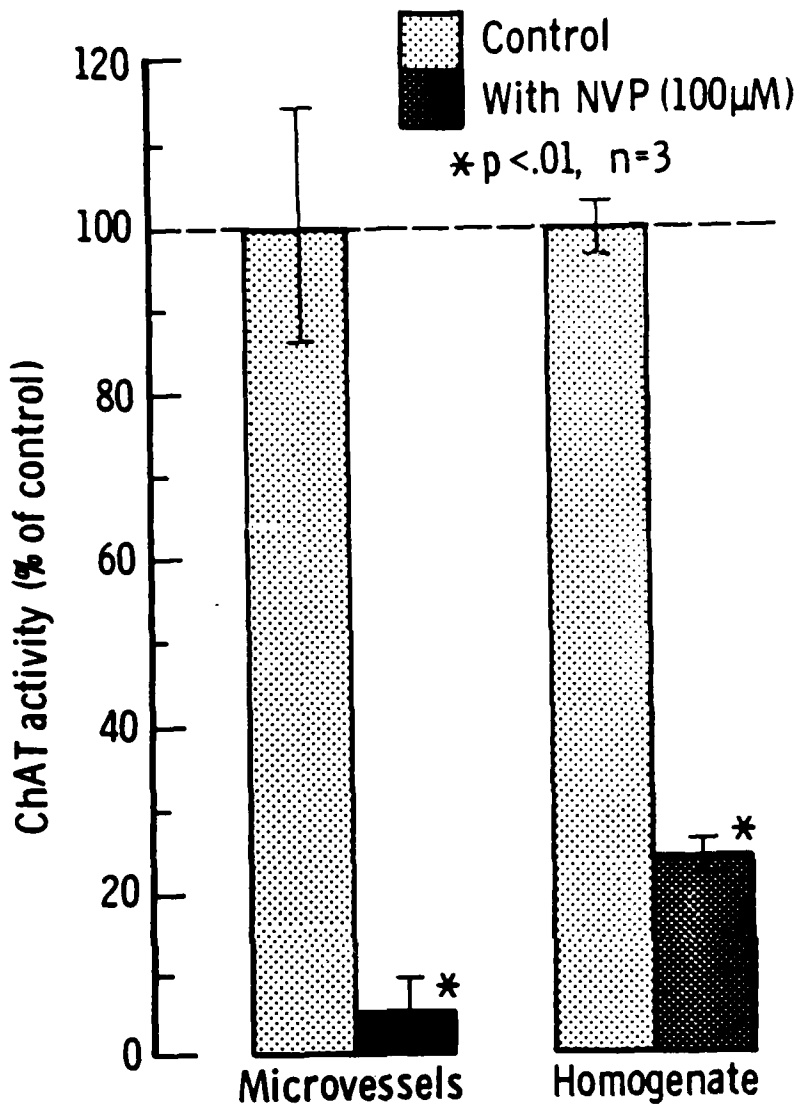


Fig. 15. The effect of the specific inhibitor of ChAT, 4-naphthylvinylpyridine (NVP, 100 uM), to inhibit the formation of ^{14}C-ACh in cortical microvessels or synaptosomes. Values are means ± S.E.M.; * indicates the value is significantly different from control, $p < 0.05$.

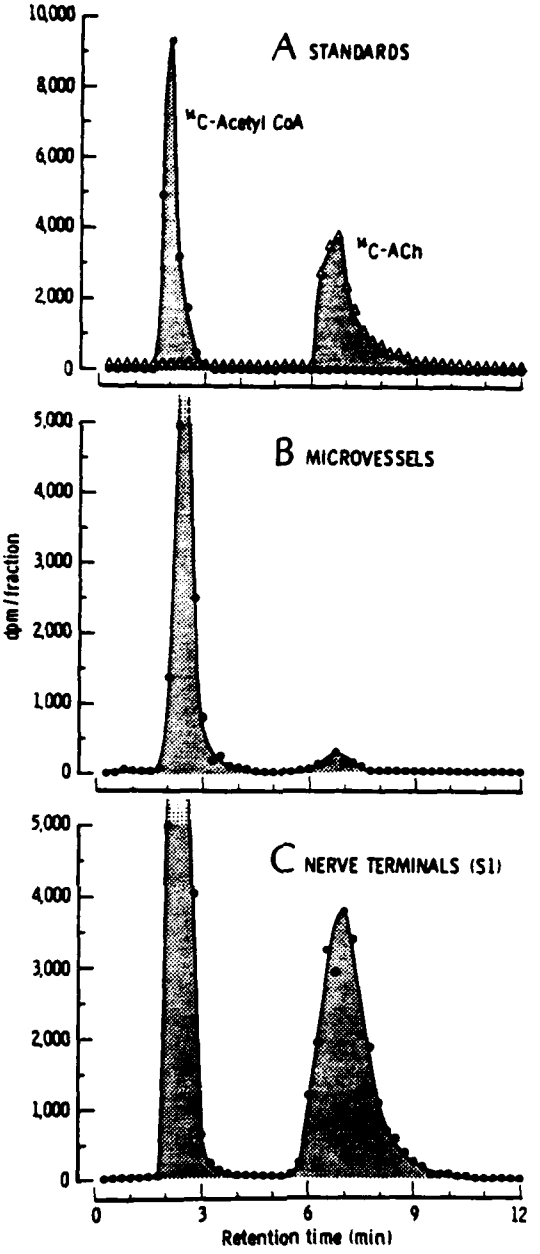


Fig. 16. Representative chromatographs depicting the separation of ^{14}C-acetylCoA and ^{14}C-ACh by the use of HPLC analysis. Peaks were detected by counting 15 sec fractions with liquid scintillation spectroscopy. Panel A—Standards containing known quantities of ^{14}C-acetylCoA and ^{14}C-ACh were injected, and the observed retention times are shown. Panel B—The formation of a small quantity of radioactive product produced from cortical microvessels that co-chromatographs with the ^{14}C-ACh standard is shown. Panel C—As expected, even more product that co-chromatographs with the ^{14}C-ACh standard is produced by the fraction containing cholinergic nerve terminals (S_1).

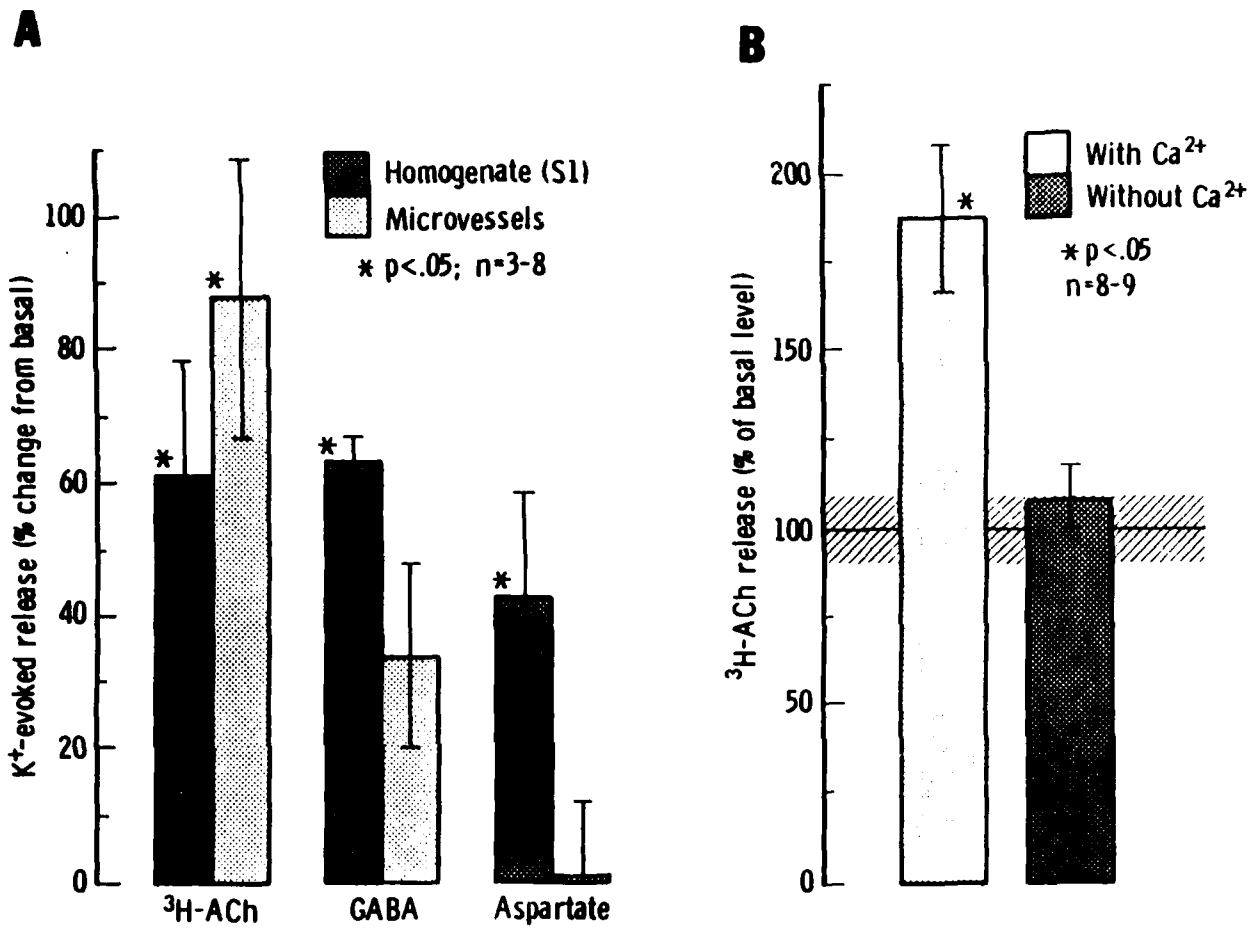


Fig. 17. Panel A—The release of ^{3}H-ACh, GABA, and Asp evoked by 55 mM K^+ in the presence of 1.2 mM Ca^{2+}, from cerebral cortical microvessels (MV) and synaptosomes (S_1). Panel B—The effect of removing extracellular Ca^{2+} on the release of ^{3}H-ACh evoked by 55 mM K^+ from cerebral cortical microvessels. Values are means ± S.E.M.; * indicates the value is significantly elevated above the spontaneous release value, $p < 0.05$.

TABLE 1

Mean Arterial Pressure (MAP), pH and Arterial Blood Gases in Rats Examined

		UNSTIMULATED			FN-STIMULATION		HYPERCARBIA
		UNOPERATED	VEHICLE	ATROPINE	VEHICLE	ATROPINE	ATROPINE
MAP	(mmHg)	124 ± 11	124 ± 6	136 ± 4	134 ± 2	130 ± 5	110 ± 6
pO_2	(mmHg)	409 ± 14	429 ± 19	431 ± 20	427 ± 10	409 ± 21	408 ± 30
pCO_2	(mmHg)	34.8 ± 0.5	35.7 ± 0.5	34.9 ± 1.0	36.5 ± 0.5	36.6 ± 0.9	59.0 ± 1.4*
pH		7.42 ± 0.02	7.41 ± 0.02	7.41 ± 0.01	7.32 ± 0.02*	7.32 ± 0.01*	7.24 ± 0.01*
N		6	6	4	11	5	5

Values represent means ± S.E.M.; animals are anesthetized, paralyzed and artificially ventilated with 100% O_2;

* $p < 0.05$, significantly different from unoperated control.

TABLE 2

pH, pCO_2 and pO_2 of Buffer Solutions Applied to the Parietal Cortex of Rats with and without FN-stimulation or with Hypercarbia.

		UNSTIMULATED		FN-STIMULATION		HYPERCARBIA
		VEHICLE	ATROPINE	VEHICLE	ATROPINE	ATROPINE
pH	R	7.40 ± 0.05	7.39 ± 0.05	7.38 ± 0.05	7.54 ± 0.05	7.63 ± 0.09
	L	7.46 ± 0.80	7.38 ± 0.05	7.35 ± 0.04	7.39 ± 0.07	7.58 ± 0.09
pCO_2 (mmHg)	R	32.7 ± 4.2	33.9 ± 1.6	31.9 ± 1.8	25.8 ± 6.0	21.8 ± 3.1*
	L	33.7 ± 3.8	33.9 ± 1.5	31.8 ± 1.6	32.9 ± 5.1	20.5 ± 2.0*
pO_2 (mmHg)	R	428 ± 77	490 ± 36	414 ± 49	394 ± 67	373 ± 72
	L	425 ± 78	508 ± 35	410 ± 49	463 ± 77	384 ± 50
N		6	4	11	5	5

(a) Values are means ± S.E.M.; * $p < 0.05$, significantly different from corresponding vehicle, unstimulated control (ANOVA).

(b) Buffer solutions were bubbled with 95% O_2: 5% CO_2, the above values determined and the solution applied to the parietal cortex. Solutions were continuously bubbled with 95% O_2: 5% CO_2 following application to the cortex.

(c) Atropine sulfate (100 uM) applied to right parietal cortex.

(d) No right-to-left differences with any treatment ($p > 0.05$; paired t-test).

TABLE 3

Effect of Bilateral Craniotomy and Atropine Sulfate (100 uM) on Resting Regional Cerebral Blood Flow (rCBF)[a,b,c]

		rCBF (ml/100 g · min)		
		CONTROL	PARIETAL CRANIOTOMY	
Region		UNOPERATED	VEHICLE	ATROPINE
Frontal Cortex	L	92 ± 8	93 ± 12	100 ± 13
	R	94 ± 9	79 ± 7	91 ± 13
Parietal Cortex	L	95 ± 7	82 ± 12	83 ± 7
	R	93 ± 5	70 ± 6	85 ± 9
Occipital Cortex	L	87 ± 8	86 ± 12	88 ± 6
	R	89 ± 7	72 ± 7	85 ± 9
Caudate Nucleus	L	92 ± 14	75 ± 6	84 ± 7
	R	82 ± 6	75 ± 6	85 ± 10
Hippocampus	L	70 ± 5	78 ± 8	72 ± 3
	R	73 ± 5	74 ± 6	78 ± 7
N		6	6	4

a) Values are means ± S.E.M.; animals are anesthetized, paralyzed and artificially ventilated with 100% O_2.

b) Atropine was applied only to the right parietal cortex.

c) No significant right-to-left differences were detected (paired t-test, $p > 0.05$); nor were there differences between treatment groups (ANOVA, $p > 0.05$).

TABLE 4

Enrichment of γ-glutamyltranspeptidase and alkaline phosphatase activities in vascular fractions as compared to gray matter

	γ-Glutamyl Transpeptidase (umol/mg prot./min)		Alkaline Phosphatase (nmol/mg protein/min)	
	Cerebral Cortex	Caudate Nucleus	Cerebral Cortex	Caudate Nucleus
Microvessels	3.7 ± 0.5	17.5 ± 4.4	85 ± 25	665 ± 90
Homogenate	0.6 ± 0.1	0.6 ± 0.1	30 ± 10	30 ± 5
Ratio	6.2	29.2	2.8	22.2

Values are means ± S.E.M.; N = 3.

TABLE 5

Rank ordering of the spontaneous release of putative neurotransmitters from cerebral cortex microvessels or homogenate

Microvessels:	**Glycine**	>	**Aspartate**	>	**GABA**	≫	**ACh**
(N=5-8)	68.3 ± 5.3		10.7 ± 1.4		3.7 ± 0.9		0.29 ± 0.05
Homogenate (S_1):	**GABA**	>	**Glycine**	>	**Aspartate**	≫	**ACh**
(N=3)	35.1 ± 4.6		22.3 ± 1.2		16.6 ± 2.9		1.0 ± 0.05

Values are means ± S.E.M. (pmol/mg protein/5 min.). Release was measured in the presence of 5 mM K^+ and 1.2 mM Ca^{2+}.

TABLE 6

A comparison of the choline acetyltransferase (ChAT) activity measured by different authors from various fractions isolated from the cerebral cortex.

Author	Species	Fractions Containing ChAT Activity			
		Intraparenchymal Vessels		Synaptosomes	Homogenate
		Size	Activity		
Arneric et al., 1986 (present study)	Rat	68% <10um 32% 10-40um	2850	2150	1475
Santos-Benito and Gonzales, 1985 (J. Neurochem. 45:633)	Rat	95% <10um	55	1435	1005
Goldstein et al., 1975 (J. Neurochem. 25:715)	Rat	95% <10um	14	--	560
Estrada et al., 1983 (Brain Res. 266:261)	Bovine	85% <10um	190	--	1118

Values are mean pmol ^{14}C-ACh formed/mg protein/min.

DISTRIBUTION LIST

1 copy	Commander US Army Medical Research and Development Command ATTN: SGRD-RMI-S Fort Detrick, Frederick, MD 21701-5012
5 copies	Commander US Army Medical Research and Development Command ATTN: SGRD-PLE Fort Detrick, Frederick, MD 21701-5012
2 copies	Defense Technical Information Center (DTIC) ATTN: DTIC-DDAC Cameron Station Alexandria, VA 22304-6145
1 copy	Dean School of Medicine Uniformed Services University of the Health Sciences 4301 Jones Bridge Road Bethesda, MD 20814-4799
1 copy	Commandant Academy of Health Sciences, US Army ATTN: AHS-CDM Fort Sam Houston, TX 78234-6100

END

DATE

FILMED

9-88

DTIC